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DIRECT INCORPORATION OF MOLECULAR OXYGEN INTO ORGANIC MATERIAL BY RESPIRING CORN SEEDLINGS[1,2]

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It is generally agreed that much of the aerobic respiration of plant tissue is accompanied by the transfer of electrons from organic substrates to molecular oxygen and the reduction of oxygen to water (3). The cytochrome system catalyzes a reaction series of this type, and the bulk of the evidence accumulated during the last several years favors the view that this enzyme system plays an important role in the respiratory oxygen consumption of plant tissue (4).

However, not all enzymes which react with molecular oxygen function in the same manner. Mason (6) recently classified the oxidases (enzymes which catalyze reactions with molecular oxygen) according to their mechanism of action; the oxidases which catalyze the addition of one molecule of oxygen to one molecule of substrate so that both atoms of consumed oxygen appear in the organic product were called oxygen transferases. Mason characterized lipoxidase as an oxygen transferase; this enzyme catalyzes the formation of peroxides of linoleic, linolenic and arachidonic acids and their esters (5).

Lipoxidase has been demonstrated in enzyme preparations from several plant materials (8), including etiolated corn seedlings (2). The existence in corn seedlings of an enzyme which catalyzes the addition of molecular oxygen to cellular constituents makes it reasonable to suppose that oxygen absorption during respiration may be accompanied by the direct incorporation of molecular oxygen into organic constituents of respiring tissue. The present work was initiated to explore this possibility.

MATERIALS AND METHODS

Grain of corn (*Zea mays* L.) hybrid Wf9 × 38-11 was soaked for 12 hours in distilled water and sowed in vermiculite (heat-expanded mica); the age of seedlings was calculated from the time of immersion in water. Soaking and subsequent germination and growth took place at room temperature in a dark chamber with occasional momentary exposures to white light. Seedlings (in groups of 6) were transplanted with wetted vermiculite to Erlenmeyer flasks and were exposed for several hours in the dark at room temperature to oxygen-nitrogen mixtures (volume ratio 20 : 80). The oxygen gas contained 10 atom percent O^{18} excess, and was prepared by electrolysis of water purchased from the Weizmann Institute of Science, Rehovot, Israel. After exposure, the endosperms were discarded, and the shoot-root axes with attached cotyledons were cut into very small pieces, frozen and dried exhaustively by means of a vacuum pump exhausting to a pressure of about 0.1 micron; the drying was continued for 12 to 15 hours.

The dried tissue was powdered in a mortar with pestle; a sprinkling of silica sand was added to facilitate grinding. Then several milliliters of chloroform were added, and the powdered tissue was extracted at room temperature by further grinding. The chloroform extract was collected by filtration. The powdered residue was washed for several hours with chloroform in a Goldfisch extractor. Oxygen in the chloroform extracts and residues was recovered quantitatively as carbon dioxide by pyrolysis in an Unterzaucher apparatus (1), and isotope ratios were determined with a Model 21-201 Consolidated Electrodynamics Corporation mass spectrometer. The normal abundance of O^{18} in seedlings not exposed to enriched atmospheres was determined for several seedlings and found to be 0.207 atom percent O^{18}.

Enriched gas mixtures used in experiments with homogenates and purified lipoxidase were the same as those applied to the seedlings. For the experiment in which seedlings were exposed to H_2O^{18} and normal oxygen, the gas phase was air.

RESULTS AND DISCUSSION

In order to test directly the hypothesis that respiration of etiolated corn seedlings is accompanied by the addition of molecular oxygen to organic materials, seedlings of several ages (0.5 day to 5.5 days) were exposed for periods of 12, 16 and 24 hours to O_2^{18}. Table I shows the incorporation of O^{18} by these seedlings. The data show that the chloroform soluble fractions contained significant amounts of O^{18}. It is also apparent that substances soluble in chloroform

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TABLE I

DISTRIBUTION OF O^{18} IN EXTRACTED FRACTIONS AFTER EXPOSURE OF SEEDLINGS TO O_2^{18}

AGE OF SEEDLINGS (DAYS)	EXPOSURE TIME (HOURS)	SIZE OF FLASK (ML)	ATOM PERCENT O^{18} EXCESS	
			CHLOROFORM SOLUBLE FRACTION	RESIDUE
0.5	16	50	0.020	....
1.5	16	125	0.056	0.018
2.0	12	250	0.194	0.044
2.0	24	250	0.366	0.042
2.0	24	50	0.096	0.018
2.5	16	125	0.106	0.036
3.5	16	125	0.090	0.014
4.5	16	125	0.064	0.022
5.5	16	250	0.084	0.022

Atom percent O^{18} excess = atom percent O^{18} in sample—normal abundance of O^{18} (0.207 atom percent). We have determined the precision of these values to be $\pm$ 0.007 atom percent O^{18} excess at the 95 % confidence level.

were enriched much more than the materials in the chloroform insoluble portions.

Experiments with homogenates confirmed and extended the findings with intact tissues. It was shown previously (2) that lipoxidase and its substrates exist in homogenates prepared by grinding 2.25-day etiolated corn seedlings in phosphate buffer (0.1 M, pH 7.7) at room temperature, and that the lipoxidase-substrate system accounted for the major portion of the oxygen absorption of these homogenates. Accordingly, an homogenate was exposed in an Erlenmeyer flask to O_2^{18} for 7 hours; oxygen saturation in the liquid phase was insured by shaking the flask in a water bath at 30° C. The chloroform extract obtained from this homogenate carried 0.188 atom percent O^{18} excess, and the residue obtained after chloroform extraction had only 0.018 atom percent O^{18} excess. Lipoxidase is not sensitive to cyanide (5) and a parallel experiment was performed in which potassium cyanide (0.002 M) was added to an homogenate; the chloroform extract carried 0.184 atom percent O^{18} excess. It is apparent from these two experiments that a cyanide-insensitive oxygen absorption by the homogenates is responsible for the bulk of the oxygen incorporated into chloroform soluble components of the homogenates.

Further experiments were performed with purified lipoxidase (purchased from Nutritional Biochemicals Corporation, Cleveland). Lipoxidase has not yet been completely and rigorously identified as an oxygen transferase (6). In the present work, it was of interest to demonstrate the ability of lipoxidase to incorporate labeled molecular oxygen into substrate. Linoleic acid (60 % purity) emulsified in 0.1 M ammonium chloride (9) was added to purified lipoxidase and oxidized in O_2^{18} at 30° C; reactions were run in Warburg respirometers so oxygen uptake could be measured. Three trials were performed and the O^{18} content of the substrate (after drying in a vacuum pump and extracting with chloroform) was determined. The O^{18} enrichment in the chloroform extract was found to be high in each trial. The maximum enrichment was 55 % of the theoretical value (5, 6); in making the calculations, it was assumed that the impurity in the linoleic acid was oleic acid. In view of the uncertainty regarding the degree of dispersion and solubilization of the fatty substrate in emulsion systems, further trials to increase the yield were not attempted. These experiments support Mason's (6) assumption that lipoxidase adds oxygen directly to substrate, and thus add weight to the conclusion that the observed incorporation of respiratory oxygen into the chloroform soluble fraction of intact etiolated corn seedlings (table I) is the result of direct addition of oxygen to chloroform soluble substrates of oxygen transferases, and that it is mediated at least in part by lipoxidase.

Even though the experiments in which seedlings were exposed to O_2^{18} (see table I) supported the view that oxygen absorption during respiration of intact etiolated corn seedlings was accompanied by the addition of molecular oxygen to chloroform soluble constituents of respiring tissue, nevertheless the possibility existed that the observed enrichment in the chloroform extract of respiring tissue was an indirect effect caused by the reduction of O_2^{18} to water during respiration followed by subsequent incorporation of H_2O^{18} into chloroform soluble components. To exclude this possibility, a control experiment was carried out in which one lot of seedlings was exposed to H_2O^{18} and normal oxygen, and another lot of seedlings was exposed to normal water and O_2^{18}. All other conditions were identical; exposure time was 16 hours and 2.5-day seedlings were used. For the exposure to H_2O^{18} and normal oxygen, seedlings were transplanted to vermiculite thoroughly wetted with H_2O^{18} containing 1.2 atom percent O^{18} excess (purchased from Stuart Oxygen Company, San Francisco). The enrichment of the chloroform soluble fraction of these seedlings was only 0.048 atom percent O^{18} excess and that of the residue obtained after chloroform extraction was 0.124 atom percent O^{18} excess. The relatively high O^{18} content of the chloroform insoluble residue indicated that the water bathing the seedlings penetrated into the tissues and that the internal, metabolically active water contained at least 0.124 atom percent O^{18} excess. This was probably a minimal value for the H_2O^{18} content of the internal water: very likely the actual value was much higher, because seedlings of this age increase rapidly in fresh weight. For the seedlings incubated in normal water and O_2^{18} on the other hand, the enrichment in the chloroform extract was 0.106 atom percent O^{18} excess and only 0.036 atom percent O^{18} excess in the residue (see table I). Also for these seedlings exposed to O_2^{18} and normal water, it could be readily calculated (knowing the fresh and dry weights and the respiratory rates of seedlings of this age) that the respiratory water produced during the incubation period would

be enriched to a maximum of 0.15 atom percent O^{18} excess, assuming that all of the respired oxygen was reduced to water and that no water within the tissues exchanged with water bathing the tissues. Thus, in these two experiments, the amounts of H_2O^{18} in the internal water were either approximately equal—or more probably, the internal H_2O^{18} content of tissues incubated in labeled water was greater than that of tissues exposed to labeled oxygen. Yet even with these internal H_2O^{18} concentrations, the chloroform extract of seedlings exposed to $O_2{}^{18}$ and normal water was enriched to more than twice the value of the chloroform extract of seedlings exposed to H_2O^{18} and normal oxygen (0.106 atom percent O^{18} excess compared to 0.048 atom percent O^{18} excess). Consequently, it seems very improbable that the O^{18} enrichment of the chloroform soluble fraction of seedlings exposed to $O_2{}^{18}$ came indirectly from H_2O^{18}, rather than directly from $O_2{}^{18}$. The experiments with purified lipoxidase and linoleic acid confirm these conclusions and offer another answer to the possible contention that reduction to water is an obligatory prelude to incorporation of O^{18} from $O_2{}^{18}$ into chloroform soluble components of these plant tissues. The cyanide insensitivity of O^{18} incorporation by homogenates exposed to $O_2{}^{18}$ also indicates that the pathway of O^{18} incorporation into the chloroform soluble fraction is not by way of water, because all of the metallo-protein oxidases which form water would be expected to be sensitive to relatively high (0.002 M) concentrations of cyanide.

It was of interest to estimate the portion of the respired oxygen which was incorporated into the chloroform soluble fraction in vivo. To determine this, 2.25-day seedlings were exposed for 24 hours to $O_2{}^{18}$ and then were dried and extracted with chloroform. One aliquot of the fraction soluble in chloroform was converted to CO_2 with the Unterzaucher apparatus and analyzed for O^{18}. A known amount of glucose was added to another aliquot so as to dilute the sample with normal oxygen, and after conversion to CO_2 in the Unterzaucher apparatus, this sample was also analyzed for O^{18}. From the observed isotope dilution, the total oxygen content of the chloroform soluble components could be calculated (7). From this information, and the value for the oxygen consumption of the seedlings over the 24-hour-period (determined with Warburg respirometers on an identical lot of seedlings), the recovery of respired oxygen in the chloroform soluble fraction could be calculated and was found to be approximately 0.4 %. This was presumably a minimal value for incorporation of respiratory oxygen into this fraction, since a portion of this incorporated oxygen probably found its way into the tissue water through subsequent metabolic processes. A portion may also have been lost through the decomposition of labile peroxides during isolation. Oxygen transferases thus account for at least 0.4 % of the oxygen absorbed in the respiration of 2.25-day etiolated corn seedlings. Present knowledge does not permit a decision as to whether this oxygen fixation is accomplished solely by lipoxidase, or whether other oxygen transferases also participate in the process.

Summary

Etiolated corn seedlings and homogenates were exposed for several hours to atmospheres containing nitrogen and oxygen enriched with O^{18}. Chloroform soluble substances recovered after exposure contained much larger amounts of O^{18} than did the residues from chloroform extraction. On the basis of this and additional supporting evidence, it was concluded that some of the oxygen consumed by the seedlings during the respiratory process was incorporated directly into organic material through the agency of enzymes which catalyze the direct addition of molecular oxygen to substrates. Lipoxidase is such an enzyme, and it was estimated that lipoxidase may participate to the extent of 0.4 % in the respiratory oxygen absorption of 2.25-day etiolated corn seedlings.

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Literature Cited

1. Doering, W. von E. and Dorfman, E. Mechanism of the peracid ketone-ester conversion. Analysis of organic compounds for oxygen-18. Jour. Amer. Chem. Soc. 75: 5595–5598. 1953.
2. Fritz, G. and Beevers, H. Lipoxidase and the oxygen absorption of homogenates from corn seedlings. Arch. Biochem. Biophys. 55: 436–446. 1955.
3. Goddard, D. R. and Meeuse, B. J. D. Respiration of higher plants. Ann. Rev. Plant Physiol. 1: 207–232. 1950.
4. Hill, R., and Hartree, E. F. Hematin compounds in plants. Ann. Rev. Plant Physiol. 4: 115–150. 1953.
5. Holman, R. T. and Bergström, S. Lipoxidase or unsaturated fat oxidase. In: The Enzymes, Chemistry and Mechanism of Action, J. B. Sumner and K. Myrbäck, eds. Vol. 2. Pp. 559–580. Academic Press, New York 1951.
6. Mason, H. S. Mechanisms of oxygen metabolism. Science 125: 1185–1188. 1957.
7. Rittenberg, D. and Foster, G. L. A new procedure for quantitative analysis by isotope dilution, with application to the determination of amino acids and fatty acids. Jour. Biol. Chem. 133: 737–744. 1940.
8. Siddiqui, A. M. and Tappel, A. L. Comparison of some lipoxidases and their mechanism of action. Jour. Amer. Oil Chem. Soc. (In press.)
9. Tappel, A. L., Boyer, P. D. and Lundberg, W. O. The reaction mechanism of soybean lipoxidase. Jour. Biol. Chem. 199: 267–281. 1952.

A STATISTICAL EVALUATION OF A GROWTH SUBSTANCE BIOASSAY METHOD USING EXTRACTS OF DORMANT PEACH BUDS[1,2]

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The techniques for separating and identifying plant growth substances have changed rapidly during the past few years. Many plant materials have been studied for their adaptability as a bioassay test (7) and paper chromatography is now being used by many investigators to separate the growth substances. With the rapid advances, using these newer methods, no recent evaluation of the sources of variation in the new techniques has been made. Although Bentley's work (2) had a marked effect on subsequent methods no advantage has been taken of her discovery that "occasion" interacted with other effects investigated; giving rise to doubt about the wide use of within vial variation as an estimate of error.

In studying the occurrence of growth substances in peach flower buds, using paper chromatography and the wheat coleoptile straight growth bioassay, there appeared to be considerable variation between samples collected on the same day. Also, it was observed that extracted growth substances failed to lodge in the same positions on successive chromatographs. The purpose of this investigation was to learn if these sources of variation affected the variation of section means and if the true experimental error was correctly estimated by coleoptile variation of coleoptiles within vials.

MATERIALS AND METHODS

Dormant Elberta peach flower buds collected January 29 and February 4, 1957, were used in this study. Immediately after each collection the buds were mixed thoroughly, divided into 3-g samples, and stored at – 12° C until they were used.

The growth substances were extracted from the buds with methanol at 3° C in the dark for 1.75 hours. The methanol extract was evaporated to dryness under reduced pressure and partially purified by a technique described by Nitsch (6) which he adapted from Jones and Reddick (5). The technique is briefly as follows: The methanol residue was taken up in acetonitrile and hexane (1 : 1) then shaken in a separatory funnel. The hexane fraction, containing some fats and pigments, was discarded, and the acetonitrile fraction reduced to dryness. The residue was dissolved in ether and acidified with 0.001 *N* HCl in a separatory funnel. The ether layer was dryed over sodium sulfate, reduced to a volume of approximately 0.3 ml and placed in a syringe for application on a paper chromatogram.

The extract was placed equally at three separate locations on the base line of the chromatographic paper. Each location or spot of extract represented 1 g fresh weight of buds. Descending chromatography on Whatman No. 1 paper was used throughout this study. The paper was equilibrated for at least 10 hours before the solvent (butanol : ammonia : water 10 : 1 : 1) was added. After the solvent had descended about 20 cm the paper was removed from the chamber and dried in the laboratory at room temperature using subdued light. After drying, the paper was cut into 17 equal sections including the original spot. For an untreated control a piece of paper the same size as the other sections was cut, usually above the base line. One exception to this is mentioned in the results section.

The pieces of paper were then placed in small vials (12 × 60 mm) with 1 ml of a phosphate-citrate buffer (pH 5.0) and 2 % sucrose. Before the coleoptiles were added the vials were shaken for 30 minutes to elute from the paper any growth substances that may be present.

The wheat coleoptile straight growth test was used for the bioassay. The variety used was Atlas 66 which was selected on the basis of uniform growth after a preliminary investigation of 12 varieties which included a variety used by other investigators (7, 9). The method is briefly as follows: The seed was soaked in water for two hours, seeded in moist sawdust, and placed in an incubating chamber in a dark room at 24° C and 90 % relative humidity. After three days, the wheat coleoptiles were ready for use. Coleoptiles 25 to 35 mm long were selected. A 3-mm section from the tip was removed and the next 4 mm (52 micrometer divisions) was used for the bioassay. The primary leaf was not removed from the coleoptile. Cutting was done with a Van de Weij coleoptile microtome[3] as improved by Thimann and described by Nitsch (7). Cutting of the coleoptiles was done under a light similar to that described by Nitsch (7), consisting of two green fluorescent tubes wrapped with three layers each of amber and green cellulose acetate window shading. After cutting, the coleoptiles were soaked in distilled water for three hours; then selected at random and placed in vials containing 1 ml of the buffer-sucrose mixture and the paper section from the chromatogram. The vials were placed on a wheel rotating at 1 rpm to reduce the amount of curvature developed during the growth period. The coleoptiles were allowed to grow for 20 hours; after which they were removed from the wheel and measured with a binocular microscope

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[2] Published with the approval of the Director of Research as paper No. 851 of the Journal Series.

[3] Coleoptile microtome obtained from E. L. Wilkie, Physics Department, Harvard University.

equipped with an eyepiece micrometer. Thirteen micrometer divisions were equal to 1 mm.

Results

Experiments were designed to detect various sources of variation together with their effect on experimental error, to determine the appropriate scale to be used for measurements, and to discover the optimum allocation of samples and coleoptiles in the experimental design.

Sources of Variation: In the 1st experiment, growth substances were extracted from four separate samples of buds collected the same day. Two of the samples were processed February 26 and the other two February 28. The chromatograms were developed and divided into sections as previously described. Ten coleoptiles per vial or section were used in the experiment. The statistical analysis (table I A) showed a smaller mean square for coleoptiles, 23, than for experimental error, 347. This means that there are sources of variation, affecting the section means, other than the variation of coleoptiles in the vials. This finding recurred in every experiment.

In all experiments conducted there were highly

Table I

Analysis of Variance and Mean Length of a Growth Substance Test Involving Four Samples, Two Processed on Each of Two Days *

A. Analysis of Variance

Source of variation	Degrees of freedom	Mean square
Samples		
Within days	2	797
Between days	1	5854
Sections (treatments)	17	3558
Experimental error	51	347
Within days **	34	335
Between days **	17	383
Coleoptiles	648	23

For error: $F = 347/23 = 15.1$, $P < 0.001$
For sections: $F = 3558/347 = 10.3$, $P < 0.001$
For samples same day: $F = 797/23 = 34.65$
For samples between days: $F = 5854/23 = 254.5$

B. Mean Length of Coleoptiles in Micrometer Units (13 units = 1 mm)

Sample	Date processed	
	2/26/57	2/28/57
1	80.5	86.9
2	78.3	83.3
Average	*79.4*	*85.1*

* Eighteen sections of the chromatograph per sample were bioassayed with ten coleoptiles per section.
** Components of experimental error. See text.

significant differences among the sections. The substance or substances extracted from the peach buds located at R_f 0.53 inhibited the growth of the wheat coleoptiles. The occurrence and properties of the inhibitor are being studied and will be reported at a later time. Histograms showing the inhibition, as well as the variation of coleoptile growth between replications, are shown in figure 1.

Table II

Analysis of Variance of a Growth Substance Test Involving Two Samples, Two Aliquots per Sample, 18 Chromatographic Sections per Aliquot, Each Section Cut in Two Pieces *

Source of variation	Degrees of freedom	Mean square	Estimate of component of variance
Samples	1	534	...
Sections (treatments)	17	7507	...
Experimental error	17	1117	53
Aliquots in sample	2	220	0
Aliquots × section in sample	34	62	0
Halves of same aliquot	4	312	3
Halves × section of same aliquot	68	89	14
Coleoptiles	576	21	21

* Five coleoptiles per vial.

The large mean square between days as compared to that within days has only one obvious explanation, the different growth potential of coleoptiles in trays prepared on different occasions. This coincides with Bentley's findings. The contrast recurred in a 2nd set of samples chromatographed on three separate days. But in both experiments the components for experimental error (within and between days) were substantially the same. Though the coleoptile growth potential is different between days, the coleoptiles used for a given treatment grew the same relative amount from day to day when comparing it with other treatments. This is indicated by the similarity of the within and between days experimental error of 335 and 383 respectively in table I.

To investigate more fully the sources of variation in experimental error, which is the failure of similar sections of paper to differ equally, another experiment was designed. The object was to measure the growth substances from two separate samples with two aliquots from each sample. The paper from each aliquot was divided in half longitudinally for half section comparisons. This provided measurements of the variation between samples, between aliquots within a sample, among sections, and among halves of sections.

The mean squares and estimates of components of variance for this experiment are indicated in table II. (A component of variance measures the variation attributable to only one particular source.) It is to be observed again that experimental error has a large component of variance which is separate from that of the coleoptiles and from those of the contributing interactions. The only interaction contributing materially to error is that between halves and sections,

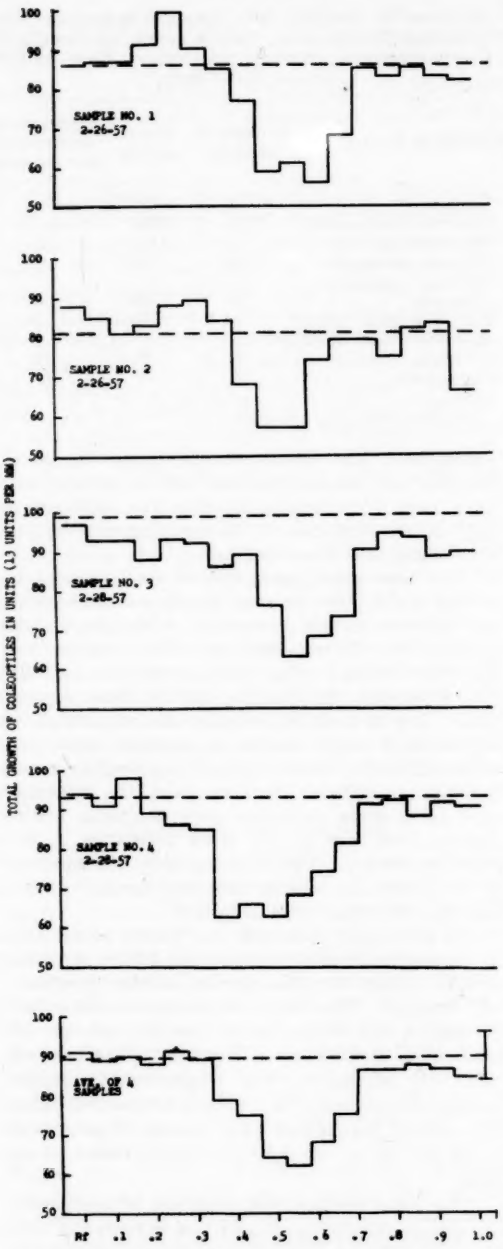

FIG. 1. Diagrams showing the lengths of wheat coleoptiles treated with eluates from the chromatogram. The original length of the coleoptiles was 52 units. The dashed lines indicate the lengths of untreated coleoptiles. The vertical bar on the lower histogram shows the 95 % confidence interval.

due apparently to irregularities in the shape of the spots in the paper.

To examine the variations in paper, known concentrations of indoleacetic acid (IAA) and indolebutyric acid (IBA) were freshly prepared and chromatographed three separate times. The chromatographic chamber was in a constant temperature room maintained at 26° C. Each chromatogram was divided into 17 equal sections and sprayed with the Gordon Weber (4) reagent. The results showed that these substances did not always occur in the same sections or have the same R_f values. In some chromatograms IAA was detected in sections 6 to 9 (R_f 0.38) in another 7 to 10 (R_f 0.42) and another 8 to 11 (R_f 0.51). The spot generally covered the same total area but varied in its position. IBA occurred in sections 11 to 13 (R_f 0.67), 12 to 14 (R_f 0.71) and 14 to 16 (R_f 0.82) a variation of three sections.

This means that known substances chromatographed on different sheets of paper and different days may not always occur in the same position. This failure of repeatability doubtless occurs with unknown as well as known substances. It may well be a major contributor to experimental error. It follows that replication of samples is essential in order to estimate correctly the true experimental error.

THE APPROPRIATE SCALE OF MEASUREMENTS: In some growth substance literature (1, 3, 8) the data have been converted to percentages of control. This makes it seem easy to compare results of different treatments in the same experiment or of the same treatment in different experiments. Difficulties arise when standard errors are needed for tests of significance. Since both section mean and control mean are variable, the standard error of their ratio is a somewhat complicated expression which varies from one section mean to another. Moreover the distribution of the percentages is not normal. Both facts raise doubts about application of the ordinary t and F tests, including confidence intervals.

If it were necessary to use percentages, ways for avoiding the difficulties would have to be found. But the results of several experiments indicate that use of the original growth increments gives valid estimates and tests as well as convenient methods of comparing means. These results are of two kinds.

First, there were three experiments in which all coleoptiles had essentially the same treatment. In each experiment the coleoptiles were incubated for the normal time (20 hours) in the buffer-sucrose solution as untreated controls. The increases in length of these coleoptiles followed approximately normal distribution curves (fig 2). Data distributed like these are appropriately treated by the ordinary statistical methods following additive models.

A 2nd kind of evidence for the usual additive methods is based on tests of additivity in analysis of variance, using Tukey's test (11). This test was applied to five experiments with F equal to 0.57, 0.05, 0.002, 4.5 and 0.0005. Non-additivity was negligibly

TABLE III

ANALYSIS OF VARIANCE OF A GROWTH SUBSTANCE TEST INVOLVING FOUR SAMPLES, 18 SECTIONS, AND TWO COLEOPTILES PER SECTION

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	ESTIMATED COMPONENT
Sample	3	292	
Sections	17	712	
Error	51	211	$\frac{211-33}{2}=89$
Coleoptiles	72	33	33

small in four of the experiments and non-significant in the 5th. The evidence is clearly in favor of additivity. This is fortunate because additivity is assumed in making the ordinary tests of significance by F or t.

It follows that in presenting growth substance data and in analyzing them statistically the actual growth units should be used. Transforming the data to percentages of control introduces unnecessary complications.

OPTIMUM ALLOCATION OF SAMPLES AND COLEOPTILES: True replication includes all sources of variation contributing to the variation of means. Thus as replications one should use individual samples carried separately throughout the bioassay procedure rather than coleoptiles treated the same within one vial.

The number of replications one should use depends on the precision desired and the funds available for the experiments. The data above suggest that since the coleoptile variance is small there is little advantage in using 10 coleoptiles per vial as most investigators have done. If the same number of coleoptiles were used with more true replications (samples), the variance of the mean and the confidence intervals might be much smaller.

The following experiment furnishes an example of this. Four samples each chromatographed and divided into 18 sections with only two coleoptiles per section were used. The analysis of this experiment, shown in table III, had 33 as an estimate of the coleoptile error component and 89 as that of the experimental error. If a new experiment with n_2 coleoptiles and n_1 samples were conducted, the components of variance being unchanged, the experimental error would be estimated as $33+89n_2$. A section mean, based on n_1n_2 observations, would then have the variance, $\frac{33+89n_2}{n_1n_2}$ or $\frac{33}{n_1n_2}+\frac{89}{n_1}$.

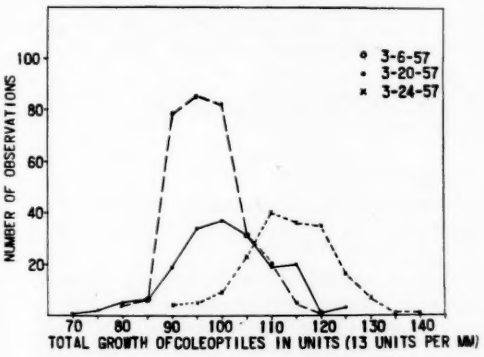

FIG. 2. Total growth of coleoptiles treated similarly on 3 days. The original length of the coleoptiles was 52 units.

It is clear that this variance, measuring inversely the sensitivity of the experiment, is little affected by n_2 (number of coleoptiles per vial), but is decreased in direct proportion to n_1 (number of samples). It is more effective to control the large component of error by increasing n_1 than to bother with the small component by increasing n_2.

Table IV shows that by using the same total number of coleoptiles but having more samples and less coleoptiles per vial the sensitivity is greatly increased. For example, substituting in the above formula, if one uses 8 samples ($n_1=8$) and only one coleoptile per vial ($n_2=1$) the variance of the mean can be reduced from 93.1 to 15.2.

So far nothing has been said about the costs of the experiments specified in table IV. Instead of basing decisions on only the variance of the mean, it is more realistic to consider what combination of samples and coleoptiles per vial will produce the smallest variance per unit cost. This depends partly on the relative costs of coleoptiles and samples.

In terms of time, the cost of processing coleoptiles (soaking and planting seed, then cutting and finally measuring coleoptiles) was estimated as $c_2=1.5$ minutes each. The additional cost of a section per sample (collecting buds, purifying, chromatographing, handling vials, etc.) was approximately $c_1=2.5$ minutes. These values, along with $s_1^2=89$ and $s_2^2=33$, may be substituted in a formula to determine the number of coleoptiles per vial (n_2) to produce the least variance for a specified total cost (10).

$$n_2=\sqrt{\frac{c_1}{c_2}\cdot\frac{s_2^2}{s_1^2}}$$

TABLE IV

ESTIMATE OF THE VARIANCE OF MEAN BASED ON NUMBER OF SAMPLES AND NUMBER OF COLEOPTILES PER VIAL

EXAMPLE	NUMBER OF SAMPLES N_1	NUMBER OF COLEOPTILES PER VIAL N_2	COLEOPTILES PER TEST	VARIANCE OF MEAN
1	1	8	8	93.1
2	1	10	10	82.3
3	2	4	8	48.6
4	4	2	8	26.4
5	4	4	16	24.3
6	8	1	8	15.2

This gives $n_2 = \sqrt{\frac{2.5}{1.5} \cdot \frac{33}{89}} = 1.43$ coleoptiles per vial

The corresponding number of samples is $n_1 = 4.74$ with resulting variance of the mean, $V\bar{x} = 23.7$. Practically, either one or two coleoptiles per vial may be used. The corresponding numbers of samples n_1 and variance $V\bar{x}$ are:

one coleoptile per vial, $n_1 = 5$, $V\bar{x} = 24.4$
two coleoptiles per vial, $n_1 = 4$, $V\bar{x} = 26.3$

Despite the slightly greater efficiency with one coleoptile per vial (using five samples) many investigators may feel safer with two (using four samples).

The number of samples may be varied at will depending on the precision required and on the available time and material.

Summary

Methanol extracts of dormant peach buds were analyzed for possible growth promoting and inhibiting substances. Paper chromatography techniques and the wheat coleoptile straight growth test were used for separation and measuring respectively of the growth substances present. A growth inhibitor (R_f 0.53) occurred in all of the peach bud samples and is being investigated further. Results of the statistical study are summarized below:

1. Replications of coleoptiles treated similarly is an underestimate of the experimental error; true replication is by use of separate samples including all sources of variation.

2. The largest component of experimental error was the failure of known and unknown growth substances chromatographed always to appear in the same section.

3. Growth of coleoptiles follows approximately a normal distribution curve indicating additivity. This is confirmed by a statistical test of additivity. Therefore, ordinary statistical techniques can be used directly without transforming the data. Changing the growths to percentages of control introduces unnecessary complications.

4. It was shown that the variance of the mean is decreased more by increasing the number of replications (samples) than by increasing the number of coleoptiles per vial.

5. For experiments of the type described, one or two coleoptiles per section are indicated as furnishing the greatest information per hour spent.

The authors wish to express their appreciation to Dr. D. D. Mason of the Experimental Statistics Department, N. C. State College, and Dr. Loyd E. Powell, Jr. of the New York Agricultural Experiment Station for the guidance received from them in the early stages of this investigation.

Literature Cited

1. Bennet-Clark, T. A. and Kefford, N. P. Chromatography of the growth substances in plant extracts. Nature 171: 645–647. 1953.
2. Bentley, J. A. An examination of a method of auxin assay using the growth of isolated sections of Avena coleoptiles in test solutions. Jour. Exptl. Bot. 1: 201–213. 1950.
3. Blommaert, K. L. J. Growth- and inhibiting-substances in relation to the rest period of the potato tuber. Nature 174: 970–972. 1954.
4. Gordon, S. A. and Weber, R. P. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192–195. 1951.
5. Jones, L. R. and Riddick, J. A. Separation of organic insecticides from plant and animal tissues. Anal. Chem. 24: 569–571. 1952.
6. Nitsch, J. P. Free auxins and free tryptophane in the strawberry. Plant Physiol. 30: 33–39. 1955.
7. Nitsch, J. P. and Nitsch, C. Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight-growth test for auxins. Plant Physiol. 31: 94–111. 1956.
8. Poljakoff-Mayber, A., Goldschmidt-Blumenthal, S. and Evewari, M. The growth substances content of germinating lettuce seeds. Physiol. Plantarum 10: 14–19. 1957.
9. Powell, L. E., Jr. A study of some non-acidic growth substances in apple leaves. Ph.D. Thesis, Cornell University, Ithaca 1955.
10. Snedecor, G. W. Statistical Methods. Iowa State College Press, Ames 1956.
11. Tukey, J. W. One degree of freedom for non-additivity. Biometrics 5: 232–242. 1949.

EFFECT OF METAL CHELATES ON GROWTH OF CORN IN SOLUTION CULTURES[1,2]

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Most of the basic studies on metal chelates in plant nutrition relate to soil applications. Not as much is known about their use in solution cultures where effects of variables such as hydrogen ion concentration, essential micro- and macro-elements and their interactions can be better controlled and studied than in soil. This paper presents results of two series of experiments with corn grown under greenhouse conditions in which chelating agents were employed in solution cultures.

Corn seeds (Kingscrost, Ke) were germinated in sand. The seedlings were transplanted to culture

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jars of 3.8 liters capacity (1 gallon) 6 days after planting, when the shoots were about 7 cm tall. The most uniform plants were selected from a large number of seedlings. Roots were thoroughly washed in distilled water before transplantation.

For each treatment, there were five jars with two plants in each (unless otherwise stated). The solutions were changed weekly and were adequately aerated once in the middle of each week. The greenhouse temperature was maintained at approximately 25° C. Plants were harvested after 21 days from the date of transplantation and dry weights of tops and roots were taken as a measure of the effects of the different treatments.

The basic composition of the solution was as follows (per liter):

Macro-salt	*Concentration in millimoles*	*Micro-salt*	*Concentration in micromoles*
Ca-acetate	0.26	H_3BO_3	8.3
$NaNO_3$	0.40	$MnCl_2$	4.0
$(NH_4)_2SO_4$	0.27	$ZnSO_4$	0.3
NH_4NO_3	0.14	$CuSO_4$	0.1
$MgSO_4$	0.33	$FeCl_3$	62.0
KCl	0.21		
Ethyl Ammonium Phosphate	0.48		

The composition of the solution is obviously different from any conventional type. It was developed to suit the purpose of this experiment. Calcium acetate was used because it acted as a buffer and helped to keep the hydrogen ion concentration of the solution fairly constant during the experiment. The use of ethyl ammonium phosphate instead of inorganic phosphates, commonly used, calls for special explanation. This compound is a water-white to yellow tinged liquid of ammoniacal odor (specific gravity 1.23 at 25° C). Chemically it is a mixture of 35 % monoethyl diammonium phosphate, 50 % diethyl ammonium phosphate and 15 % monoethyl ammonium hydrogen phosphate. Thirty eight percent of this compound is phosphorus as P_2O_5. Unlike inorganic compounds of phosphorus, it does not form precipitates of iron phosphate with ferric chloride. Moreover, this organic compound was found to be not only physiologically non-toxic, but also a good source of phosphorus in nutrient culture.

The 1st experiment was concerned with the effects of the disodium salt of EDTA [3] on corn seedlings, grown in solutions as given above, except for the varying amounts of EDTA. The 2nd experiment was concerned with the substitution of chelated magnesium ("Versenol" magnesium chelate) for the usual source, magnesium sulfate.

The results of the 1st experiment are shown in table I. It appears from our results that there is a definite correlation between the amounts of EDTA in solution and plant growth, which was progressively better with decreasing concentrations. Amounts of 80 micromoles or higher were sooner or later lethal to the plants. At 200 micromoles concentration plants died immediately after transplantation. There was no increase in dry weight at this concentration. For this reason the weights of these plants were taken as a base and were subtracted from the mean dry weights in all other treatments to show the mean dry weight increase (columns 5 and 6, table I). Plants receiving 160 micromoles of EDTA survived for about a week, whereas those receiving 120 micromoles and 80 micromoles died in about 8 and 12 days respectively from the date of transplantation. At 40 micromole and 20 micromole levels plants were very chlorotic and stunted. Concentrations of 10 micromoles, 5 micromoles and 2.5 micromoles not only were non-toxic, but appeared to be growth-promoting as compared to the control which contained no EDTA. It seems from this that the upper non-toxic level of EDTA concentration approximated 10 micromoles per liter. It also may be noted that plants receiving non-toxic concentrations of EDTA grew more luxuriant root systems than those without this compound.

[3] EDTA: Ethylene diamine tetraacetic acid.

TABLE I

EFFECT OF DIFFERENT CONCENTRATIONS OF EDTA ON THE GROWTH OF CORN IN SOLUTION CULTURE

Na_2EDTA micromoles per liter	Mean dry wt g/plant		Standard error		Mean dry wt increment after transplantation		Mean dry wt increment as % of control	
	Top	*Root*	*Top*	*Root*	*Top*	*Root*	*Top*	*Root*
0 (control)	1.53	0.61	0.109	0.045	1.40	0.49	100	100
2.5	1.90	1.02	0.096	0.062 **	1.77	0.89	126.7	182.4
5.0	1.75	0.91	0.088	0.094 **	1.62	0.79	115.7	160.5
10.0	1.67	0.91	0.109	0.045 *	1.54	0.78	110.2	160.3
20.0	0.67	0.43			0.54	0.31	38.9	63.0
40.0	0.40	0.28			0.27	0.15	19.4	31.3
80.0	0.29	0.23			0.16	0.11	11.7	21.7
120.0	0.18	0.16			0.05	0.04	3.8	7.4
160.0	0.14	0.13			0.01	0.01	0.9	1.4
200.0	0.13	0.12			0.0	0.0	0.0	0.0

* Difference compared with control significant at 0.05 level.
** Difference compared with control significant at 0.01 level.

The chelating agent (Na_2EDTA) when mixed with a solution of various inorganic salts, forms complexes with metals in an order depending on the stability of the corresponding complexes. It forms more stable compounds with iron and copper than with any other metals, and chelated iron has been successfully used as a source of iron in solution culture (5, 6, 7). It may be noted, however, that at all levels of Na_2EDTA below 20 micromoles the molar ratio of EDTA to Fe^{+++} was less than 1 : 1. This means that at these levels all the Fe^{+++} ions were not chelated. Therefore, Fe from $FeCl_3$ rather than from Fe-EDTA was probably the more dominant source of iron for the plants. Besides, the supply of iron was adequately insured even without the addition of EDTA to the solution since ethyl ammonium phosphate did not form precipitates of iron phosphate with ferric chloride. Therefore, the beneficial effect of low concentrations of EDTA on plants growing in solution culture must be attributed to a growth-promoting property of this compound.

Heath and Clark (3, 4) have suggested that EDTA at very low concentrations acts as a growth substance and that 3-indole acetic acid affects growth in the same way as a chelating agent. Gross (2) reported an increase in activity of adenosine triphosphatase at lower concentrations of EDTA, but a significant inhibition in its activity at higher concentrations. Weinstein et al (8) found that 10 ppm of EDTA is optimum for soybeans grown in solution cultures. Results by Jacobson (5) on corn were in substantial agreement with this.

There is some evidence from the work of Gross (2) that chelates in low concentrations may provide some protection to enzyme systems from traces of heavy metals. Whether this would be a factor in the beneficial effect of low concentrations of EDTA on plants grown in culture solutions is a question needing further investigation.

In the 2nd experiment, chelated magnesium ("Versenol" magnesium chelate) replaced magnesium sulfate in the culture solutions (table II). Dunn and Roberts (1) found no beneficial effect of magnesium Versene [4] on corn and apple seedlings grown in solution cultures. Previous work in the Horticultural Department of the University of New Hampshire by R. Eggert (unpublished data) showed that Mg-EDTA in high concentrations was very toxic to plants either as a foliar spray or in soil applications.

In this experiment there were 10 culture jars per treatment with two plants in each jar. Except as noted in table II the solutions used were identical in composition with those used in the control of the previous experiment. Plants were harvested 21 days from the date of transplantation.

Treatment A was considered to be the control, and the plants received magnesium sulfate and "Versenol" iron chelate as sources of Mg and Fe. Plants in both treatment A and B looked very vigorous as compared to those of treatment C which were distinctly stunted and had all the symptoms of magnesium deficiency (chlorosis, necrotic leaf spots, poor root growth, etc.). The dry weight yields of tops and roots in treatment B were found to be significantly higher than those of treatment C.

[4] Magnesium Versene: Sequestrene Na_2Mg of Geigy Chemical Corporation.

TABLE II

ROLE OF VERSENOL MAGNESIUM CHELATE AS A SOURCE OF MAGNESIUM FOR CORN IN SOLUTION CULTURE

TREATMENTS, MICROMOLES PER LITER	MEAN DRY WT G/PLANT		STANDARD ERROR	
	Top	*Root*	*Top*	*Root*
A. Versenol Fe-chelate, 9 micromoles (instead of $FeCl_3$)	0.94	0.33	0.015 **	0.006 **
B. Versenol Mg-chelate, 6 micromoles (instead of $MgSO_4$), Versenol Fe-chelate, 4.5 micromoles (instead of $FeCl_3$)	1.05	0.35	0.152 *	0.016 **
C. No magnesium and Versenol iron chelate, 9 micromoles (instead of $FeCl_3$)	0.55	0.25	0.034	0.008

* Significant at 0.05 level over treatment C.
** Significant at 0.01 level over treatment C.
Versenol iron and magnesium chelate are metals chelated with "Versenol" (Dow Chemical Company) which is a trisodium salt of *N*-hydroxy ethyl ethylene diamine triacetic acid.

The following points may be considered in explanation of the results. The total concentration of "Versenol" metal chelate in any of the treatments did not exceed 10.5 micromoles which was found to be the optimum concentration for "Versenol" under these conditions. The "Versenol" magnesium chelate in treatment B should dissociate in the solution to a very negligible extent throughout the experiment. This is because both iron and magnesium were in chelate form and there would be no possibility for the iron to replace the magnesium from its complex. The percentage of magnesium present as metal in the "Versenol" magnesium chelate, however, is considerably lower than that in magnesium sulfate (treatment A). In spite of this low concentration of magnesium in treatment B, the plants did not show any signs of magnesium deficiency (with treatment C as an index). Instead they were just as vigorous as those in treatment A. This indicates that the magnesium of the "Versenol" chelate was as readily utilized by plants as that of magnesium sulfate. There appears to be no other published account of the successful use of chelated magnesium as a plant nutrient in solution culture.

SUMMARY

Ethylene diamine tetraacetic acid in low concentrations (10 micromoles and below) was distinctly

beneficial to plant growth, especially to root development, in nutrient cultures under greenhouse conditions.

The magnesium salt of "Versenol", if supplied in non-toxic concentrations and with proper adjustment of the solution composition, can substitute successfully for magnesium sulfate as a source of magnesium for corn grown in solution cultures.

We wish to thank Mr. E. J. Haertl of the Dow Chemical Company, for his advice and for furnishing certain of the compounds used in this investigation. Thanks also are due the Monsanto Chemical Company for supplying the sample of ethyl ammonium phosphate.

Literature Cited

1. Dunn, S. and Roberts, Suzanne S. The availability of magnesium from organic carriers in artificial substrates. Plant Physiol. 29: 337–347. 1954.
2. Gross, M. Effect of EDTA on adenosine triphosphatase activity. Science 118: 218–219. 1953.
3. Heath, O. V. S. and Clark, J. E. Chelating agents as plant growth substances: a possible clue to the action of auxin. Nature 177: 1118–1121. 1956.
4. Heath, O. V. S. and Clark, J. E. Chelating agents as growth substances. Nature 178: 600–601. 1956.
5. Jacobson, L. Maintenance of iron supply in nutrient solution by a single addition of ferric potassium ethylene diamine tetraacetate. Plant Physiol. 26: 411–413. 1951.
6. Stewart, J. and Leonard, C. D. Chelates as sources of iron for plants growing in the field. Science 116: 564–566. 1952.
7. Weinstein, L. H., Robbins, W. R. and Perkins, H. F. Chelating agents and plant nutrition. Science 120: 41–54. 1954.
8. Weinstein, L. H., Meiss, A. M., Uhler, R. L. and Purvis, E. R. Effect of ethylene diamine tetraacetic acid on nitrogen metabolism and enzyme patterns in soybean plants. Contrib. Boyce Thompson Inst. 18: 357–370. 1956.

THE WATER CONTENT OF MAPLE STEMS. I. APPLICATION OF THE KARL FISCHER METHOD FOR THE ANALYSES OF WATER IN MAPLE BARK AND WOOD[1,2]

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In physiological studies in which water determinations are a problem the ovendry method may have definite limitations. In such experiments alcohol extraction of the water followed by titration with Karl Fischer reagent is a useful alternative method. Karl Fischer first reported in 1935 on the reagent which now bears his name (3). Since then the very general usefulness of this reagent in analyses for water has been recognized. Mitchell and Smith (14) have critically reviewed the literature describing the various applications of the Karl Fischer titration. In the determination of moisture in native wood samples, Mitchell (13) obtained good agreement between the titration and ovendry methods. In general he found higher values with the Fischer titration. His data are for samples of air-dried Douglas fir, kiln-dried cypress and partly-dried oak. No data on the water content of fresh wood as determined by the Karl Fischer method were available.

Although references to analyses for water in wood using the Fischer method are few, there is a considerable amount of published data on the moisture content of wood, determined by oven-drying or by other methods. These data relate to two distinct problems concerning water content; either its relation to the physical and mechanical properties of wood as lumber, or its relation to the physiology of the living tree. As concerns lumber, McMillen (12) states that even though wood must have proper moisture content for maximum utility, none of the standards associations has adopted a standard testing procedure. When water content must be determined the industrial wood users depend on oven-drying.

The method of sampling fresh wood for water determinations becomes more of a problem in studies of tree physiology. McDermott (11) using the ovendry method, showed that the percent moisture of tree branches varied significantly with the method of cutting the samples. His samples were taken when the water in the xylem elements was presumably under tension; i.e., under conditions of rapid transpiration. Cutting breaks this tension, causing the water to pull away from the cut end. Determinations on such samples gave significantly lower values than those on samples where a simultaneous cutting method prevented the removal of any of the water. Gibbs (4) had previously considered the possibility of changes during sampling and avoided it by using a method of punching out cores of wood. In a later report (6) Gibbs considered McDermott's findings in relation to a natural gradient in the water content along the branch. He confirmed McDermott's results and found that natural gradients were too small to affect them.

Another sampling problem originates from a difference in water content of successive annual rings or between sapwood and heartwood. Huber (7) presented data from Langer on the wood, water, and

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air content in 55 growth rings of fir, showing a decrease in water content with an increase in age. Similar data on water content only for 8 growth rings in *Eucalyptus regans*, with both early and late wood included, were presented by Stewart (15). Jones and Bradlee (8) determined water content of sugar maple sampled each month for 25 successive months. A statistical analysis of their data showed that inner wood (heartwood) had a significantly lower water content than either outer wood (sapwood) or top wood.

Seasonal changes in water content are another source of variation which should be considered when taking samples. Gibbs (5) and Clark and Gibbs (2) have investigated the seasonal patterns of water content of several tree species, with reference to the practical problem of flotation and sinkage of logs. Maximum water content was at leaf opening, minimum at leaf fall. Campbell and French (1) gave seasonal moisture on a fresh weight basis for normal oak sapwood to compare with that of sapwood infected with oak wilt fungus. They found significant differences in water content due to "seasons," namely periods of dormancy, budding, growing, and resting. The highest water content occurred during the growing season.

The seasonal change in water content in sugar maple was followed by Jones and Bradlee (8). A statistically significant variation due to months was calculated from their data. Water content was highest in the winter. It decreased during the sap flow period, reached a minimum during the growing season, and then increased to the winter maximum. K. N. H. Greenidge determined the water content of maple wood sampled each month from October to June. His data (kindly furnished to the authors in a personal communication) showed a significant variation due to month with the highest monthly average in December.

Besides varying with season and between sapwood and heartwood or successive annual rings, water content may vary with location in the length of the tree. Clark and Gibbs (2) found significant differences in the water content at different levels in the trunk of the conifers: balsam fir, red spruce, Eastern hemlock, and Eastern white cedar. They found no significant differences in water content at different trunk levels in several species of birch. Calculations on the data of Jones and Bradlee showed no difference in the water content of sapwood and topwood. No vertical moisture gradient could be demonstrated using the data of Greenidge.

In the experiments reported here, the Karl Fischer method was applied to the determination of the water content of living dormant maple wood and bark from small excised stems. A specialized requirement of these experiments which this method fulfilled was the determination of apparent water content at below freezing temperatures. Such data were needed to test an hypothesis that changes in the temperature of maple bark and wood, as from -5 to 25° C, caused changes in their apparent water content, namely, in the amount of alcohol-extractable water, and that such changes could be related to the sap flow phenomenon. The method was first "standardized" against oven-drying with determinations of water in bark and wood at room temperatures. Under such conditions excellent agreement between methods has been shown. A difference between results with the two methods when used on frozen maple bark and wood has been demonstrated.

Since the review of the literature indicated several possible types of natural variation among samples, large numbers of replicate determinations were considered essential. The Fischer method, being much faster, was adapted to this requirement. In so doing a method of taking small replicate samples of either bark or wood was developed. This method, which can be used for either frozen or thawed material, is described.

Materials and Methods

The water content data presented here were determined from samples of dormant maple bark and wood. The problem of water movement during sampling of leafy stems (4, 6, 11) is not important in this case since the samples were taken from dormant stems. A "conditioning" treatment, described later, was also found to reduce sampling errors. The samples, taken from maple stems 2 to 3 cm in diameter and 60 cm long, were cut with a spokeshave adjusted to cut a strip the thickness of the bark. The curved blade of the spokeshave had been ground out to leave a tongue in the middle which cut the sample 0.5 cm wide. Long strips of bark cut in this manner were immediately cut with scissors to lengths of 2.5 cm and two such pieces were put into a weighed graduated test tube containing 10 ml of absolute methanol and closed with polyethylene stoppers. The tubes were then reweighed. The fresh weight of the samples varied from 100 to 200 mg. Wood samples were cut in the same manner, immediately after the bark had been removed with a spokeshave having a normal curved blade. Wood samples were of the same thickness as bark samples and contained only wood from the most recent growth rings.

After four hours were allowed for the extraction of the water by the methanol, a 1- or 2-ml aliquot was removed by pipette and the amount of water was determined by titration with Karl Fischer reagent in a Beckman aquameter. At least two aliquots from each sample were run. The results of these replications were considered acceptable only if they differed by less than 0.05 ml of titrant. For each set of analyses a blank determination was run on the methanol. The Karl Fischer reagent was standardized prior to each experiment by titration against the primary standard, C.P. sodium tartrate dihydrate. A water-in-methanol standard was not used because of its tendency to pick up atmospheric moisture. The water content was calculated as percent on a dry weight basis.

Samples for oven-drying were taken by the same cutting method, put into weighing bottles with standard-taper ground glass tops, weighed, and dried to constant weight at 100° C. The samples from frozen material were cut in the cold room at – 5° C and immediately put into cold methanol.

Large variation in the moisture content of samples taken from the same stem necessitated running large numbers of samples from each given experimental condition of the stem. Twelve to 15 samples were taken in most cases. Means and fiducial limits were calculated in the usual way.

Results

Factors Affecting the Extraction: Since these experiments represented a new application of the Karl Fischer method, some of the factors which might affect the results were studied. Determinations of the rate of extraction of the water by the absolute methanol showed that after four hours without agitation, the amount of water extracted reached a plateau and remained constant thereafter. A second treatment with fresh methanol extracted only a very small additional amount of water. The amount was constant so a second extraction was not used. No difference was found in the amount of water in samples which were shaken or stirred during extraction. Interfering substances which might dissolve from the bark or wood and react with the titrating reagent were found to be absent or, in some cases, present in amounts barely discernible with the method.

Comparison with Oven-drying: Comparisons between the Fischer and ovendry methods were made (table I). The 28 samples of bark used in these experiments were all taken from the same stem. They were taken alternately, one sample for titration, the next for oven-drying. The mean percent water for 14 samples of frozen maple bark, determined by titration, was 50.11. The mean for 14 samples of frozen maple bark, determined by oven-drying was 54.36 % (table I, part A). In the "after run" condition, i.e., after a section of stem was warmed from – 5° to 25° C and a flow of sap had occurred, the mean of the Fischer method determinations was 61.84 %, while oven-drying gave a mean percent of 62.98. Thus the amount of water found in these samples of frozen maple bark depended on the method by which it was determined. For determinations on material at room temperature no difference between methods was indicated.

When similar experiments were run on maple wood (table I, part B) a mean percent of 56.37 was obtained by the Fischer method on samples taken while frozen. Oven-drying gave a water content of 66.15 % on samples of wood from the same frozen stem. Thawed wood gave results of 57.02 % and 58.33 %. The same conclusions regarding the methods can therefore be made for determinations on maple wood as for those on maple bark; namely, the determined values depend upon the method and not the material.

Application of the Method: Having established that the Karl Fischer method was applicable to determinations of water in frozen bark and wood, several determinations were made on sections of single stems of maple. These stems had previously been "conditioned," a treatment based on the findings of Marvin and Erickson (10). After cutting, any time after leaf fall and before the end of the spring sap flow season, the stems were brought to room temperature, then cooled over a 24-hour period to 1° C. While cooling, the cut ends were immersed in a 3 % sucrose solution. They were then frozen, while still in contact with the sucrose solution, and stored at – 8° C. This conditioning treatment produces a more

Table I

Comparison of the Karl Fischer and Ovendry Methods of Determining Water Content of Maple Bark and Wood *

Frozen (– 5° C)		After run (25° C)	
Karl Fischer	Ovendry	Karl Fischer	Ovendry
Part A. Percent water, dry matter basis, in maple bark			
51.98	57.58	56.89	60.98
50.08	54.77	65.45	57.11
54.15	55.84	56.42	64.37
46.86	56.08	60.13	70.91
52.02	56.13	64.66	60.31
54.46	50.29	66.50	66.31
49.68	54.75	61.84	58.40
45.56	49.14	60.05	57.56
48.48	50.58	66.03	63.69
45.20	54.23	61.73	66.64
46.71	59.01	65.65	60.90
57.58	50.92	60.80	67.70
46.07	54.56	59.64	59.54
52.77	57.18	60.03	70.71
		....	59.57
Mean 50.11	54.36 **	61.84	62.98
Std. error 1.03	0.81	0.90	1.19
Part B. Percent water, dry matter basis, in maple wood			
52.71	66.31	58.53	61.92
45.60	64.27	63.19	58.35
68.57	75.78	66.17	55.79
52.39	68.23	66.31	48.39
59.71	49.37	54.34	58.30
65.50	80.03	60.57	54.13
50.06	57.16	52.79	66.75
55.98	67.13	52.30	57.73
54.73	58.73	55.87	71.70
62.10	57.55	56.20	53.68
60.12	65.00	53.35	57.70
53.65	68.42	49.21	53.73
51.67	69.52	57.95	55.67
....	81.32	51.38	62.76
....	63.50	....	....
Mean 56.37	66.15 **	57.01	58.33
Std. error 1.80	2.23	1.45	1.58

* Mitchell (13) has shown the Karl Fischer method to be accurate to two decimal places. The variation in the material used here suggests that in these data the decimal places have no biological significance.

** Difference between means for the two methods significant at the 5 % level.

TABLE II

PERCENT WATER IN MAPLE BARK AND WOOD, FROZEN AND AFTER A FLOW, ON REPLICATE SAMPLES FROM ONE STEM

	BARK % WATER, D.M.B.		WOOD % WATER, D.M.B.	
	FROZEN −5° C	AFTER RUN 25° C	FROZEN −5° C	AFTER RUN 25° C
	57.43	47.51	72.74	51.38
	57.01	52.35	71.50	50.74
	63.43	45.41	65.67	55.55
		48.41	75.32	53.70
	54.70	51.06	73.85	49.97
	51.65	50.35	97.36	54.46
	50.58	51.03	73.25	49.81
	60.62	45.52	68.98	51.24
	55.62	47.49	74.70	46.22
	55.42	46.07	73.91	55.35
	57.70	48.92	73.28	50.44
	53.70	47.89	58.96	49.01
	49.90	46.76	59.85	53.07
	53.56	....	....	48.10
	59.97	....	....	....
Mean	55.96	48.37 *	72.26	51.36 *
Std. error	0.98	0.63	2.58	0.74

* Difference between the means significant at the 1 % level.

uniform sap flow from the stems when they are warmed. Samples for the determination of the water content of frozen bark and wood were taken from one section of a conditioned stem. Another section of the same stem was warmed to room temperature and allowed to stand until the flow had occurred and nearly ceased. Samples were then taken for water analyses after a run. Table II contains the data from such a series of experiments. The bark showed a very significant change in water content, decreasing from 55.96 % while frozen to 48.37 % after the run. An equally significant change was found in the wood; the water content decreased from 72.26 to 56.36 %. Data from a similar series of experiments have been presented by Marvin (9) and further experiments are reported in the following paper.

DISCUSSION

Standard analytical techniques sometimes do not yield precisely the kind of information desired and less well known methods must then be used. Instead of the standard ovendry method, the Karl Fischer method for the determination of water has been used in the present study. The titration method requires no heating and therefore could be used to test the initial hypothesis that there is a temperature-induced change in the water content of maple stems between −5 and 25° C. It would not be possible to study this physiological effect using the ovendry method since the water determinations must be made at the lower as well as at the higher temperature.

The data (table II) demonstrated that there was a change in apparent water content. Both bark and wood showed a decrease in water content at 25° C from that at −5° C. However the data from table I indicated that a higher water content in frozen material was found when the ovendry method was used. This would have caused an error in the direction of bigger changes than those found when the Karl Fischer method was used.

The variation in water content of the stems used in these experiments seemed unduly large. For example, supposedly replicate samples of frozen maple wood ranged from 58.96 to 97.36 % water, d.m.b. (dry matter basis) (table II). However, results from the oven-drying experiments on frozen maple wood ranged from 49.37 to 81.32 % (table I, part B). Despite these ranges, the distribution of the replicate determinations in each experiment around their mean was shown by a t-test of symmetry to be normal. No relationship between the location on the stem whence the sample came and the water content of that sample could be found. Therefore the large variation among replicates was considered a characteristic of the material. The decrease in the apparent water content of both maple bark and wood which occurred with a temperature rise is greater than this natural variation. The relation of this change to the sap flow phenomenon is discussed in the following paper.

SUMMARY

1. The Karl Fischer titration has been applied to the determination of water in living maple bark and wood.

2. A method for obtaining more uniform samples of maple bark and wood has been developed.

3. The water content data found with the Fischer method were comparable to those found with oven-drying when the samples were taken at room temperature. The water content data from the two methods differed when samples were taken at −5° C.

4. A sizable decrease in the alcohol-extractable water content of maple bark and wood occurred with a rise in temperature from −5 to 25° C.

LITERATURE CITED

1. CAMPBELL, R. N. and FRENCH, D. W. Moisture content of oaks and mat formation by the oak wilt fungus. Forest Science 1: 265–270. 1955.
2. CLARK, J. and GIBBS, R. D. Studies on tree physiology. IV. Further investigations of seasonal changes in moisture content of certain Canadian forest trees. Can. Jour. of Bot. 35: 219–253. 1957.
3. FISCHER, K. A new method for the titrimetric determination of the water content of liquids and solids. Z. angew. Chem. 48: 394–396. 1935. Chem. Abstr. 29: 6532.
4. GIBBS, R. D. Studies of wood. II. On the water content of certain Canadian trees and on changes in the water-gas system during seasoning and flotation. Can. Jour. of Research 12: 727–760. 1935.
5. GIBBS, R. D. Studies in tree physiology. I. General introduction. Water content of certain Canadian trees. Can. Jour. of Research 17: 460–482. 1939.

6. GIBBS, R. D. Studies in tree physiology. III. The effect of the method of cutting on the water content of twigs. A note on a paper by McDermott. Can. Jour. of Research 20: 236–240. 1942.
7. HUBER, B. Die Gefasslietung. In: Encyclopedia of Plant Physiology. III. Water relations of plants. Pp. 563. Springer-Verlag, Berlin 1956.
8. JONES, C. H. and BRADLEE, J. L. The carbohydrate contents of the maple tree. Vermont Agr. Expt. Sta., Bull. 358: 1–147. 1933.
9. MARVIN, J. W. Investigations of the sugar content and flow mechanism of maple sap. Tappi 40: 209–216. 1957.
10. MARVIN, J. W. and ERICKSON, R. O. A statistical evaluation of some of the factors responsible for the flow of sap from the sugar maple. Plant Physiol. 31: 57–61. 1956.
11. MCDERMOTT, J. J. The effect of the method of cutting on the moisture content of samples from tree branches. Amer. Jour. Bot. 28: 506–508. 1941.
12. MCMILLEN, J. M. Methods of determining the moisture content of wood. U. S. Forest Products Lab. R-1649. 8 pp. 1950.
13. MITCHELL, J., JR. Determination of moisture in native and processed cellulose. Ind. Eng. Chem., Anal. Ed. 12: 390–391. 1940.
14. MITCHELL, J., JR. and SMITH, D. M. Aquametry. Pp. 1–444. Interscience Publishers, New York 1947.
15. STEWART, C. M. Status of cambial chemistry. Tappi 40: 244–256. 1957.

THE WATER CONTENT OF MAPLE STEMS. II. APPARENT CHANGES IN THE WATER CONTENT OF MAPLE STEMS AND THE PHYSIOLOGY OF MAPLE SAP FLOW[1,2]

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For many years the mechanism producing a vessel sap pressure in Acer and some other genera has attracted the attention of a few investigators. Early studies demonstrated that a rise in the temperature of dormant stems from just below or just above freezing, often induced vessel sap pressures and a flow from a wound in the stem. A decrease in stem temperature reduced the pressures and the flows.

The pressure-inducing mechanism was recognized as a characteristic of the stem by Jones et al (1) and by Wiegand (7). Jones et al (1) measured vessel sap pressures and found that during the cooling part of the diurnal cycle these pressures were below one atmosphere. They also studied the direction of the pressure and found that during a flow the larger pressure was in a downward direction. The pressure-inducing mechanism was also studied by Stevens and Eggert (6). They observed the absorption of water by excised stems and recognized that the role of the roots was to supply sap to the stem during the cooling period. Marvin and Greene (5) measured the amounts of absorption and flow by excised stems under conditions of controlled temperature changes. The quantitative relationship between temperatures and sap flow in intact plants was studied by Marvin and Erickson (4). They presented evidence that a quantitative relationship of temperature to the flow could be shown for the cooling part of the diurnal temperature cycle but not for the warming part.

Several important questions concerning the sap flow mechanism remain unanswered. Of these, two are of interest here. They are, what cells or tissues of the stem store the water and what mechanism or mechanisms hold the water during the cooling part of the sap flow cycle. The xylem of the stem may be the reservoir for the water absorbed from the roots or some of this water might move into the phloem. In such a case the phloem would serve as the reservoir for water until it moved back to the xylem where it would produce the vessel sap pressure and a flow on the warming part of the sap flow cycle. The mechanism holding the water may depend on osmotic forces, with the result that the water is held as mobile free water, or colloidal forces may act as part of the water-holding capacity of the tissues. Hydration and dehydration of colloidal materials would then be the source of the changes observed in water content following a temperature change. Information on these points could be obtained if there are differences in the alcohol-extractable water (free water) content of stems between the frozen state at the end of an absorption period and the warmed state at the end of a flow period. Information could also be obtained on where the water is held by making separate and simultaneous determinations of the water content of the bark and sapwood. The materials and procedures used have been described in detail in the preceding paper, and data on the relation between water content and flow response are presented here.

RESULTS

Significant differences were found between the extractable water content of both bark and wood, frozen and thawed. The data for replicate samples

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[2] Vermont Agricultural Experiment Station Journal Series Paper No. 77. This paper is also based upon work supported in part by grants from the General Foods Corporation and the Northeastern Forest Experiment Station of the U. S. Forest Service.

TABLE I

WATER CONTENT OF MAPLE BARK AND WOOD, FROZEN AND AFTER A RUN *

STEM NO.	PERCENT WATER, DRY MATTER BASIS			
	BARK		WOOD	
	FROZEN −5° C	AFTER RUN 25° C	FROZEN −5° C	AFTER RUN 25° C
1	44.80	54.33 ***	70.63	61.36 ***
2	56.79	64.58 ***	87.13	68.58 ***
3	55.96	48.37 ***	72.26	51.36 ***
4	46.88	38.46 ***	78.71	64.54 ***
5	45.44	51.61 **	52.11	52.82
6	56.77	55.84	71.34	55.54 ***
7	57.29	56.04	78.75	70.37 ***
8	68.47	64.09	75.27	71.15
9	64.18	56.08	68.84	55.02 ***

* Each value is the mean of 12 to 15 determinations.
** Difference between percent water frozen and after run significant at 5 % level.
*** Difference significant at 1 % level.

from one stem were given in the preceding paper. Table I gives results from samples from 9 individual stems. Each value is the mean of the 12 to 15 replicate samples taken from the stem under the given conditions. Considering the bark data first, there were three stems in which water content after the thaw was higher than that of the preceding frozen condition and there were two stems in which water content after thawing was lower than before. In the other four stems no significant difference was found. Thus different stems under the same experimental conditions gave inconsistent results from the determinations of bark moisture. Results of water content determinations on wood were consistent and the water content after thawing was less than that when frozen in 7 cases. In two other cases no change was observed. These stems (No. 5 and 8, table I) were the only ones of the 9 that did not flow on warming. Thus we see that there may be an increase or a decrease in the alcohol-extractable water content of the bark under the experimental conditions that produce a simultaneous decrease in the alcohol-extractable water content of the xylem of the same stem.

It is possible that the decrease in the content of alcohol-extractable water following warming could be explained by the loss of water from the stem as a flow of sap. Using the percentages given in table I and the weights of the xylem and bark, the volume change in apparent water content was calculated. These values and the observed flows are given in table II. The decrease in water content from frozen to thawed wood may be as much as 10 times the magnitude of the flows observed, and is always greater than the flow. It would appear that there is only an apparent loss of water and that something has happened to the water in the wood such that a much larger percentage is extractable by alcohol while the wood is frozen than after it is thawed and a flow has occurred. It should also be pointed out that this change in the amount of alcohol-extractable water cannot be explained by a loss of water from the stem to the roots for the stems were excised from their roots before the experimental treatments. Attention should again be called to trees number 5 and 8. These are the same trees which gave no change in alcohol-extractable water and no flow (table I).

TABLE II

COMPARISON OF CHANGE IN ALCOHOL-EXTRACTABLE WATER CONTENT AND VOLUME OF FLOW OF INDIVIDUAL MAPLE STEMS

STEM NO.	STEM WT	CALCULATED CHANGE IN WATER CONTENT		FLOW	TOTAL CHANGE
		WOOD	BARK		
	g	*g*	*g*	*ml*	*g*
2	297	+1.2	−15.2	0.2	−13.8
3	270	−1.2	−18.8	2.0	−18.0
4	367	−2.0	−15.4	1.5	−15.9
5	290	+1.1	None	None	+ 1.1
6	256	None	−13.2	1.8	−15.0
7	210	None	− 4.9	4.3	− 0.6
8	260	None	None	None	None
9	238	None	−10.9	2.0	− 8.9

TABLE III

WATER CONTENT OF MAPLE BARK AND WOOD AT 1° C AND AT 25° C *

STEM NO.	STEM WT	FLOW	PERCENT WATER, D.M.B.			
			BARK		WOOD	
	g	*ml*	*@ 1° C*	*@ 25° C*	*@ 1° C*	*@ 25° C*
10	381	1.0	58.79	65.60	56.52	61.38
11	318	1.2	52.77	46.59 **	41.36	33.87 **
12	376	4.0	48.88	43.72	46.36	45.94

* Each value is the mean of 12 to 15 determinations.
** Difference between the means of the percent water at the two temperatures significant at the 5 % level.

Marvin and Greene (5) have shown that it is not necessary to freeze maple stems before they will flow. However, Marvin and Erickson (4) recognized that with a preceding freeze stems give much larger flows. Using this information it might be possible to evaluate the possible role of alcohol-extractable water in the sap flow mechanism. Three different stems were cooled to 1° C then warmed to 25° C. The weight and sap flows were recorded and the alcohol-extractable water determined for bark and wood at each temperature. All three stems gave a flow of sap but in only one case was there a statistically significant change in the water content of the bark and wood. This difference was significant at the 5 % level (table III). It would appear from these data that a cause and effect relationship does not exist between the change in alcohol-extractable water content and the sap flow mechanism. The decrease in alcohol-extract-

TABLE IV

FLOW CHARACTERISTICS OF MAPLE STEMS WITH AND WITHOUT BARK

STEMS WITH BARK		STEMS WITHOUT BARK		
STEM WT	FLOW	STEM WT	1ST FLOW	2ND FLOW
g	*ml*	*g*	*ml*	*ml*
409	5.2	244	4.1	1.8
186	5.0	318	11.0	4.5
192	7.8	167	1.4	0
197	12.1	271	5.9	3.2
300	15.6	265	9.2	1.9
246	7.5	164	7.2	2.1
375	11.1	229	6.9	1.3
231	4.8	246	7.9	2.4
400	12.3	323	21.0	0.6
248	8.7	171	7.6	3.7
268	6.6	234	2.0	0.1
242	6.4	145	5.5	1.3
268	12.0	226	3.2	0.3
147	7.2	317	7.2	6.2
230	7.8	274	4.1	0.6
226	8.4	216	10.8	2.8
204	5.9	197	7.8	2.3
238	3.3	308	14.2	2.0
343	2.1	131	4.7	1.5
310	4.7	215	1.2	Trace
269	12.2	254	12.0	5.3
286	12.1	252	6.6	3.5
459	4.3	160	4.5	2.5
234	2.8	236	8.6	1.8
	Mean 7.75		*Mean* 7.61	

able water at the time sap flow occurs may quantitatively affect the flow, however.

From table I it was evident that the bark, that is, the tissues external to the cambium except for the cork, gave inconsistent alcohol-extractable water content data. Also there was no evidence on the role of the phloem as a reservoir for water between the time of the water absorbing or conditioning part of the diurnal temperature cycle and the warming and sap flow part of the cycle. The role of the phloem was studied by conditioning a number of stems and removing the phloem from a section one half the length of each stem before the flow was induced. The data in table IV show that the flow from the sections without phloem were no different in amount from those of the sections with phloem. On reconditioning, the stems without phloem ran a second time. This suggests that the phloem does not act as a reservoir in the sense that it is an essential part of the sap flow mechanism.

Sugar maple stems appear, from the data just presented, to be consistent in showing marked changes in the content of alcohol-extractable water in the wood following thawing. It is of interest to know whether this temperature response occurs also in other species of Acer, other genera that do flow sap and still others that do not. Table V gives the results from water content determinations on the bark and wood of a number of plants selected as examples of these groups. *Acer pensylvanicum* L. behaved as does *Acer saccharum* Marsh. *Juglans cinerea* L. gave a flow of sap and also showed marked changes in alcohol-extractable water. *Betula lutea* Michx. which often flows sap in the spring but later than the sugar maple and butternut, did not yield a flow of sap but did show a significant change in water content in one case and not in the other. *Fraxinus americana* L. surprisingly yielded a slight sap flow but showed no significant change in water content. Negative results were obtained with *Quercus rubra* L. It would appear that the phenomenon of a temperature induced change in the content of alcohol-extractable water of wood and bark is not restricted to sugar maple.

DISCUSSION

Marked changes have been found in the alcohol-extractable water content of maple wood and bark when frozen stems are warmed to room temperature. The changes in the wood are consistent, i.e., the water

TABLE V

WATER CONTENT OF FROZEN AND THAWED BARK AND WOOD OF SEVERAL GENERA

SPECIES	STEM WT G	AMT FLOW ML	PERCENT WATER, D.M.B.			
			BARK		WOOD	
			FROZEN −5° C	THAWED 25° C	FROZEN −5° C	THAWED 25° C
Acer pensylvanicum	(a) 158	Trace	87.96	83.43	124.69	104.42 *
Juglans cinerea	(b) 637	11.4	95.20	66.30 **	141.81	100.76 **
" "	(c) 126	1.5	75.83	80.52	135.95	95.82 **
Fraxinus americana	(b) 322	1.0	43.76	41.10	36.65	35.55
Betula lutea	(b) 277	0	46.61	44.00	58.19	50.48
" "	(c) ...	0	65.30	48.43 **	73.20	60.32 **
Quercus rubra	(c) ...	0	75.10	72.88	63.29	63.92

* Difference between percent water frozen and thawed significant at 5 % level.
** Difference significant at 1 % level.
(a) Absorbed sucrose solution during conditioning (cf. preceding paper).
(b) Cut during the flow season, no pretreatment.
(c) Cut while frozen, perfused with 3 % sucrose solution before conditioning.

content decreases, while the changes in the bark are inconsistent, the water content may increase or in another stem decrease on warming. The observed changes cannot be explained as a movement of water from xylem to phloem or from stem to root. In the first instance the water content of both tissues was determined simultaneously and in the latter case the stems were excised from the roots. It would appear that since there is more alcohol-extractable water in the frozen state, some structures or materials are more hydrated in the warmed condition and are dehydrated in the frozen state.

Klenkova (2) studied the hydration of a number of plant materials, pine wood, cellulose, and cellulose derivatives, and determined by a calorimetric method the freezable and non-freezable water content after various treatments. She found that living pine wood collected in the winter had more non-freezable water than pine wood collected in the summer. Also repeated freezing increased the capacity of the wood to hold water against freezing. This non-freezable water is considered by Klenkova to be colloidally bound water. In the results presented here (table I) the alcohol-extractable water is presumably non-bound water. The data of Klenkova (2) and that presented here both show changes in the amount of bound water held by woody tissues following freezing. The results in the two sets of data are, however, diametrically opposed to each other, one giving an increase in bound (non-freezable) water, the other an increase in non-bound (alcohol-extractable) water, when the experimental tissues were held at low temperatures in contrast to tissues held at a higher temperature. No data are available to show whether a comparison of results obtained by these two divergent methods is valid or not. Seasonal temperature induced hydrations and dehydrations as related to winter hardiness have been extensively studied and were reviewed by Levitt (3).

It is of interest to note from these experiments that on warming the maple wood has a reduced amount of alcohol-extractable water at the time a flow occurs, this reduction in the content of alcohol-extractable water is much greater than the amount of water lost in the flow. Thus a vessel sap pressure is formed simultaneously with an overall decrease in the free water of the sapwood. It appears that the simultaneous sap flow and dehydration are correlated processes but may well be in opposition to each other quantitatively. Further evidence for the independence but relatedness of the change in alcohol-extractable water content and the sap flow is found in the experiments presented in table III. Without a freeze a change in the water content of the wood and bark may or may not occur following cooling and warming, but in all these stems there was a slight flow. Previous work (4, 5) indicates that the volume of a flow without a preceding freeze is less than that of a flow preceded by a freeze.

The rapid hydration and dehydration observed in sugar maple bark and wood is not peculiar to sugar maple but occurs in other species of Acer and other genera of woody plants that have a flow of vessel sap. It may well be that other genera that do not flow sap will show the rapid change in alcohol-extractable water, but this did not happen in the ash and oak stems tested.

Summary

1. A marked decrease in the alcohol-extractable water content of excised maple stems was observed when frozen stems were thawed.

2. Sap flows occur simultaneously with the decrease in alcohol-extractable water, but the latter are as much as 10 times the magnitude of the former.

3. The temperature induced change in the alcohol-extractable water in maple stems is correlated with the maple sap flow but is not an essential part of the mechanism.

4. The bark is not essential to the sap flow mechanism.

5. The decrease in alcohol-extractable water occurred in another species of Acer and in butternut and birch, other species that yield sap as a result of vessel sap pressures.

Literature Cited

1. Jones, C. H., Edson, A. W. and Morse, W. J. The maple sap flow. Vermont Agr. Expt. Sta. Bull. 103: 1–184. 1903.
2. Klenkova, N. I. Freezing of wood. III. Zhur. Priklad. Khim. 27: 539–545. 1954. Consultants Bureau Translation Pp. 499–504. Chem. Abstr. 48:9687c.
3. Levitt, J. The hardiness of plants. Academic Press, New York 1956.
4. Marvin, J. W. and Erickson, R. O. A statistical evaluation of some of the factors responsible for the flow of sap from the sugar maple. Plant Physiol. 31: 57–61. 1956.
5. Marvin, J. W. and Greene, Mary T. Temperature-induced sap flow in excised stems of Acer. Plant Physiol. 26: 565–580. 1951.
6. Stevens, C. L. and Eggert, R. L. Observations on the causes of the flow of sap in red maple. Plant Physiol. 20: 636–648. 1945.
7. Wiegand, K. M. Pressure and flow of sap in the maple. Amer. Naturalist 40: 409–453. 1906.

EFFECTS OF SOME ESSENTIAL OIL VAPORS ON THE STOMATA OF EUPATORIUM AND MENTHA[1]

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Following Tyndall's observations in 1862 (8) on the great absorptive power shown by certain essential oils of plants for thermal radiations, a number of theories have been put forward concerning the possible function of such oils in the water relations of plants secreting them.

Detto (2) tested the effect of menthol vapor on the rate of water loss from twigs of Syringa. He found that the rate was depressed, and that this was accompanied by the death of a large area of leaf surface. He also tested the effects of other oils on other plants, and came to the conclusion that essential oils played no part in regulating water loss from plants.

Dixon (3) found that the vapors given off from leaves of *Artemisia Absinthium* brought about a reduction in the transpiration rate of leafy branches of Syringa and Cytisus. He was of the opinion that such oil vapors retarded the rate of water loss either by depressing the activity of the protoplasm or by retarding the rate of diffusion of water vapor from the mesophyll cells.

The author (4) compared the effects on transpiration by some plants and evaporation from a moist filter paper of eucalyptus, peppermint and lemon oil vapors. These were found to bring about a slight depression in the rate of evaporation, but a more pronounced decrease in the rate of transpiration.

The author (5) also tested the effects of lemon, eucalyptus, peppermint and citronella oil vapors on water loss from atmometers. Vapors of fresh samples of all these oils materially reduced the rate of water loss. After a short time, however, these oils decreased in effectiveness, apparently because the more volatile fractions have been evaporated. It was suggested that essential oil vapors might cause surface tension effects leading to a reduction in the rate of evaporation.

Audus and Cheatham (1) tested the effects of some essential oil vapors on the transpiration rate, taking the rate of water absorption by cut shoots of cherry laurel as a direct measure of transpiration. On exposure of shoots to air saturated with oil vapors, they failed to detect any measurable effect on the absorption rate. The authors, however, reported a small depression which they regarded as presumably due to a toxic action of the vapor. They concluded that essential oils play no part in regulating water loss.

Again, in view of the conflicting reports and conclusions concerning the effects of essential oils on water relations of plants, the author (6) reinvestigated the effects of eucalyptus, lemon, peppermint and citronella oil vapors simultaneously on transpiration and absorption by plants. On applying these oil vapors to cut branches of Mentha and Eupatorium—one plant which did and one which did not produce essential oils themselves—the transpiration and absorption rates were significantly depressed.

On the grounds of the observation that the depressions were, however, greater in transpiration by plants (6) than in water loss by atmometers (5), it was suggested that oil vapors might have, in addition to their physical effects on evaporation of water, a possible effect on the stomatal mechanism, and that such effect would in turn affect transpiration.

The present investigation is an attempt to test this suggestion by studying the effects of the same oil vapors on the stomata of the same species of plants used in the foregoing studies (6).

MATERIAL, METHOD AND EXPERIMENTAL RESULTS

In the following experiments, two glass porometer cups of 12.5 and 7 mm internal diameters respectively were used with *Eupatorium granulosum* and *Mentha spicata* leaves. The cups were of the thistle funnel shape (fig 1), the mouth being surrounded by a rubber tube. Immediately behind the tube was inserted a side-tube which served as an inlet for a steady current of dry air. The air within the cup was therefore not subject to changes in humidity or in oxygen or carbon dioxide tensions. In order to keep the air passing evenly over the leaf surface without dead spaces, the inside nozzle of the side-tube was drawn out and bent towards the mouth of the cup.

Air was drawn through the leaf into the porometer cup by connecting the free end of the latter to a head of water in a narrow-bore horizontal tube with a short vertical arm dipping into a reservoir of cooled boiled water. Thus a constant pressure difference of 7.5 cm of water was obtained throughout the present

FIG. 1. Porometer cup, see description in text.

[1] Received revised manuscript November 26, 1957.

TABLE I

HALF-HOURLY MEANS OF 5-MINUTE POROMETER READINGS SHOWN BY EUPATORIUM GRANULOSUM WITHIN 1ST AND 2ND HALF-HOUR EXPOSURES TO OIL VAPORS, EXPRESSED AS PERCENTAGES OF INITIAL RATES JUST BEFORE TREATMENTS *

EXPERIMENT NO.	OIL CONC MG/L	FLOW RATE WITHIN 1ST ½ HR	FLOW RATE WITHIN 2ND ½ HR	EXPERIMENT NO.	OIL CONC MG/L	FLOW RATE WITHIN 1ST ½ HR	FLOW RATE WITHIN 2ND ½ HR
	Eucalyptus				*Lemon*		
1	12.0	49.0	42.8	5	10.6 (F) **	53.2	41.7
2	15.0 (F) **	62.8	56.2	6	7.6	73.0	41.6
3	10.5	59.5	....	7	8.4	35.0	....
4	12.5	61.0	....				
Totals	...	232.3	99.0	..	...	161.2	83.3
Means	...	58.1	49.5	..	...	53.7	41.7
% Depression	...	41.9	50.5	..	...	46.3	58.3
	Peppermint				*Citronella*		
8	7.7	61.0	55.0	12	15.3 (F) **	42.8	61.2
9	2.0 (F) **	80.5	....	13	5.9	82.5	65.0
10	11.5 (F) **	41.5	60.2				
11	6.1	56.0	....				
Totals	...	239.0	115.2	..	...	125.3	126.2
Means	...	59.8	57.6	..	...	62.7	63.1
% Depression	...	40.2	42.4	..	...	37.3	36.9

* Data from 13 experiments.
** (F) = A fresh sample of the oil is applied.

series of experiments. The rate of flow of the air was measured by the rate of movement of the water meniscus, and this rate was taken as an indication of the degree of opening of the stomata.

Two three-way taps were inserted between the cup and the horizontal capillary tube. One of these (t_2) served to place the capillary tube in communication either with the cup (while a porometer reading was to be taken) or with the exterior (as when the water column in the capillary tube needed to be sucked back to its original position after readings). By means of the other tap (t_1), the porometer cup could

TABLE II

HALF-HOURLY MEANS OF 5-MINUTE POROMETER READINGS SHOWN BY MENTHA SPICATA WITHIN 1ST AND 2ND HALF-HOUR EXPOSURES TO OIL VAPORS, EXPRESSED AS PERCENTAGES OF INITIAL RATES JUST BEFORE TREATMENTS *

EXPERIMENT NO.	OIL CONC MG/L	FLOW RATE WITHIN 1ST ½ HR	FLOW RATE WITHIN 2ND ½ HR	EXPERIMENT NO.	OIL CONC MG/L	FLOW RATE WITHIN 1ST ½ HR	FLOW RATE WITHIN 2ND ½ HR
	Peppermint				*Eucalyptus*		
14	4.8	96.3	91.4	17	13.2	99.7	56.0
15	10.1 (F) **	72.6	73.0	18	12.0	65.5	61.5
16	4.7	83.0	47.2	19	14.4 (F) **	41.2	49.3
Totals	...	251.9	211.6	..	...	206.4	166.8
Means	...	84.0	70.5	..	...	68.8	55.6
% Depression	...	16.0	29.5	..	...	31.2	44.4
	Lemon				*Citronella*		
20	15.6	35.2	39.1	25	12.4	31.7	....
21	16.3	52.0	....	26	13.5 (F) **	68.0	56.0
22	13.2 (F) **	111.2	111.4	27	5.5	97.3	79.0
23	8.1	62.5	....				
24	9.1 (F) **	70.5	31.2				
Totals	...	331.4	181.7	..	...	197.0	135.0
Means	...	66.3	60.6	..	...	65.7	67.5
% Depression	...	33.7	39.4	..	...	34.3	32.5

* Data from 14 experiments.
** (F) = A fresh sample of the oil is applied.

be introduced into the air-sweep circuit between readings.

By a suitable arrangement, the circulating dry air could be charged, at will, with a known weight of the oil vapor tested.

In conducting an experiment, the lower surface of a selected leaf detached under water and with its petiole dipping in water was attached to the porometer cup with rubber grease. The leaf was held in position by merely pressing it lightly against the greased edge. Dry air was circulated through the cup at a rate of four liters per hour. After a period of time, with the leaf in the diffuse daylight of the laboratory, the rate of flow of water meniscus in the porometer tube was measured at 5-minute intervals. For a state of equilibrium to be reached before taking a reading, 20 seconds were allowed to elapse after placing the column of water in communication with the porometer cup. The time required for the meniscus to move 1 cm was then determined. The air current was thus interrupted for periods of roughly one half minute while the porometer readings were taken. The leaf was next illuminated by a 500-watt lamp with the filament about 30 cm from the leaf. A cooling screen of water, changed every 15 minutes, was placed between the lamp and the leaf in order to cut off the greater part of the heat. The light intensity at the leaf surface, as measured by a photo-electric meter, was found to be 1200 ft-c.

When the new porometer readings seemed to have attained a reasonably steady value, the circulating air current was charged with oil vapor at a known concentration. After a further period of time during which 5-minute readings were taken, the oil vapor was cut off and pure air again allowed to circulate through the cup.

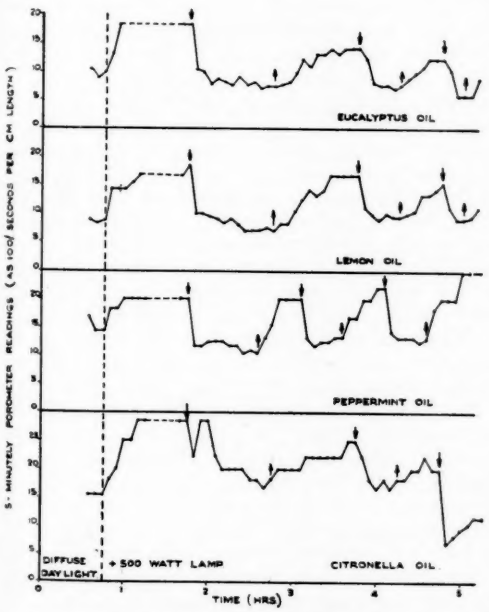

FIG. 2. Graphs showing the effects of the vapors of the four different oils upon the stomata of *Eupatorium granulosum*. (Data from experiments 1, 5, 8 and 13.) N.B. ↓ = Oil vapor on; ↑ = Oil vapor off.

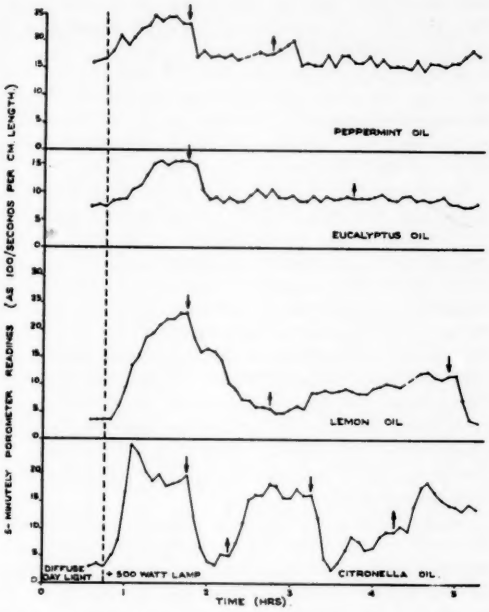

FIG. 3. Graphs showing the effects of the vapors of the four different oils upon the stomata of *Mentha spicata*. (Data from experiments 15, 18, 24 and 25.)

The temperature in the immediate vicinity of the leaf was determined by a thermometer placed very close to it.

Tables I and II show sets of half-hourly mean flow rates, as percentages of initial reference rates immediately before applying oil vapors, of 5-minutely porometer readings within the 1st and 2nd half-hour exposures of Eupatorium and Mentha respectively to eucalyptus, lemon, peppermint and citronella oil vapors.

To illustrate the action of each vapor on each plant representative experiments have been chosen and shown in graphical form in figures 2 and 3. In these figures, the individual 5-minute readings, rather than their respective half-hourly mean rates, of the representative experiments are plotted against time. Porometer readings taken at 5-minute intervals give rates of flow of meniscus in the porometer tube in arbitrary units as 100/seconds per cm length. Broken lines indicate that some readings were not taken. Allowance was made for this in calculating half-hourly mean rates.

Preliminary experiments on Eupatorium and Mentha in the absence of any oil treatment have shown

that the drift of porometer readings with time within the period of an experiment was not significant at the 5 % probability level.

Considering the data of tables I and II, it is evident that the stomata of Eupatorium and Mentha leaves are responsive to the application of the vapors of all four oils tested, the oil treatment bringing about, in each case, a distinct closing effect.

In the case of Eupatorium, a one hour exposure of the leaf to eucalyptus or lemon vapor resulted in a reduction of the stomatal pores to as little as one half or even less of their original area. The vapor effects were fully exercised even upon exposure to the lowest concentrations, obtained by merely passing the air over the oil surface (table I, experiments 3 and 6). The treatment with peppermint and citronella oils also brought about similar, though relatively lesser, closing effects. These could be detected even upon exposure to as low a concentration as 2 mg of vapor per liter of circulating air, obtained, in experiment 9, by passing the air over only one drop of peppermint oil.

In the case of Mentha, the stomata again showed the usual closing effect upon exposure to any oil vapor, the closing response being most with eucalyptus and lemon, then citronella and peppermint least. In experiment 22, the treatment with a relatively high vapor concentration (13.2 mg/liter) of a fresh sample of lemon oil gave peculiar results. The stomata immediately closed but within 10 minutes reopened to slightly more than their original areas, the apertures then remaining constant throughout the rest of the period of the one hour treatment. On cutting off the vapor, a slight still further opening of the stomata occurred followed after two hours by a gradual closing movement. In experiment 21, with still higher vapor concentration (16.3 mg/liter) but with a used sample of the same oil applied for only one half hour, a distinct closing response occurred, followed by a partial recovery upon cutting off the vapor. But, after 1.5 hours, on reapplying the vapor for another half an hour, an appreciable further opening rather than closing of the stomata occurred. It is probable that such effects are due to a toxic action of the vapor.

Concerning the post-treatment effects, the two plants behaved differently. In the case of Eupatorium, stomatal recovery of the oil-treated leaf usually started immediately on cutting off the vapor and was, in many cases, eventually almost complete. On the other hand, in the case of Mentha the closing response due to applied vapors was usually maintained either indefinitely or for a time after the vapors were cut off. This implies that a lasting or a temporary effect of oil treatment may occur. Recovery was in no case complete.

In order to test the significance of the observed responses to oil treatments, it seemed that, owing to the differences between the half-hourly means of each set (tables I and II), the best estimate of the population variance is that based on the "residual" sum of squares of deviations, i.e., the deviations of the individual (5-minute) observations about their respective (half-hourly) means. The t test of significance was applied to the following differences: (a) The difference between the grand mean of the 1st half-hour observations and the mean of all half-hourly means (adjusted to 100) just before applying the oil vapor. (b) The difference between the grand mean of the 2nd half-hour observations and the mean (= 100) just before treatment.

The conclusion was that the observed depressions in the rates of air flow through the stomata of Eupatorium and Mentha leaves within the 1st and 2nd half-hour treatments with vapors of any of the four oils tested are highly significant at the 1 % probability level. They cannot, therefore, be ascribed to chance or experimental error.

As far as any generalization can be made with reference to these experiments only, it would appear that the stomata of the plant producing essential oil itself (Mentha) are less affected by treatment with oil vapors than those of the plant which does not produce essential oil (Eupatorium). Moreover, the stomata of the plant producing an essential oil (Mentha) are less affected by treatment with its own oil vapor than by treatment with the vapors of oils produced by other plants.

The nature of the closing response of stomata to oil treatments is obscure, but these responses, at least as far as relatively high concentrations of the oil vapors are concerned, might be due to toxic action of the vapor on the leaf cells. No visible change of color was observed in the present series of experiments, but the earlier observations made by the author (4, 6), Detto (2), and Audus and Cheetham (1) may be recalled. All these authors reported that vapors of certain essential oils, when applied in high concentrations to plants, exercise harmful effects, including discoloration, drop of turgor and death of large areas of treated leaves. On the grounds of these observations, there is reason to suggest that the closing responses and failure, under certain conditions, to recover might be due to alterations in the turger pressure of the guard cells resulting in turn from toxic effects of the vapors on the guard cell membranes.

These findings on the responses of stomata to oil treatments are, however, not in line with the general conclusions arrived at by Audus and Cheetham (1). Nevertheless, these authors reported a slight closing of stomata on exposure of a cherry laurel leaf to the saturated vapor of thyme oil. Except for one experiment with rosemary oil, which also showed a transitory closing of the stomata, all the other experiments with rosemary and anise oil vapors failed to elicit any stomatal response. The reason for this failure seems likely to be due to toxic action on the leaf cells following prolonged exposures to saturated oil vapors. Reference should be made here to the abnormal results of experiments 21 and 22 (table II) of the present work. In these experiments, a one hour exposure to relatively high concentrations of

lemon vapor resulted in slight further opening of the stomata of Mentha rather than the usual closing response following brief exposure or exposure to relatively lower concentrations of the same oil. Moreover, the experiments of Audus and Cheetham are seemingly examples of stomata in a confined space, where their behavior cannot be regarded as normal. It has been shown (Williams (9); Heath and Williams (7)), that the stomata of illuminated leaves open much wider than elsewhere if the leaf surface is enclosed in a small volume of still air, as in the classical type of porometer cup used by these authors.

Summary

1) Experiments were performed to investigate the effects of eucalyptus, lemon, peppermint and citronella oil vapors upon the stomata of Eupatorium and Mentha.

2) On charging the air current circulating through the porometer cup with oil vapors, significant closing responses by the stomata of both plants could be detected.

3) Presumably the closing responses, at least as far as relatively high vapor concentrations are concerned, may be due to toxic action of the vapors on leaf cells.

Literature Cited

1. Audus, L. J. and Cheetham, A. H. Investigations on the significance of ethereal oils in regulating leaf temperatures and transpiration rates. Ann. Bot. N.S. 4: 465–483. 1940.
2. Detto, C. Die Bedeutung von atherischen Olen bei Xerophyten. Flora 92: 147–199. 1903.
3. Dixon, H. H. Transpiration and the ascent of sap in plants. Macmillan, London 1914.
4. Hafez, M. G. A. An analysis of the influence of ethereal oils on transpiration. M.Sc. thesis, Cairo University, Cairo 1939.
5. Hafez, M. G. A. Effects of some essential oil vapours on evaporation and absorption by atmometers. Proceedings of the Science Arab Congress, 3. 1957.
6. Hafez, M. G. A. Effects of some essential oil vapours on transpiration and absorption by Eupatorium and Mentha. Proceedings of the Science Arab Congress, 3. 1957.
7. Heath, O. V. S. and Williams, W. T. Studies in stomatal action. Adequacy of the porometer in the investigation of stomatal aperture. Nature 161: 178–179. 1948.
8. Tyndall, J. On the absorption and radiation of heat by gaseous matter. Trans. Roy. Soc. (London) 152: 59–98. 1862.
9. Williams, W. T. Shock-induced stomatal movements. Nature 160: 364–365. 1947.

EFFECTS OF ROSEMARY AND THYME OIL VAPORS ON THE STOMATA OF CHERRY LAUREL[1]

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The possible effects of essential oil vapors upon stomata were first investigated by Audus and Cheetham (1), and later by the author (2). Audus and Cheetham tested the effects of rosemary, thyme and anise oil vapors on the stomata of cherry laurel leaves, measuring their resistance to mass flow of air within an ordinary permanently attached porometer cup. They reported a slight closing of the stomata on exposure to thyme oil, but failed to detect any stomatal response to anise and rosemary vapors. Their conclusion was that the vapors of all these oils had no significant effects on the stomata of cherry laurel.

Using a modified type of porometer cup through which air was kept circulating, the author (2) studied the effects of eucalyptus, lemon, peppermint and citronella oil vapors upon the stomata of *Eupatorium granulosum* and *Metha spicata*, Linn. The rates of air flow through the stomata of both plants were shown to be significantly depressed on charging the air stream with oil vapors. This depression was a clear indication of a closing stomatal response following oil treatments.

There existed, however, the possibility that lack of accord between the general conclusions arrived at separately by Audus and Cheetham and the author was due to a difference in the technique by which the results of both were obtained. It is now known that the behavior of stomata within the usual form of porometer cup used by Audus and Cheetham cannot be regarded as normal (Heath and Williams (3)). Consequently it was decided to reinvestigate the effect of some of these oil vapors on the same plant used by these workers, but employing modified forms of porometer cups.

[1] Received August 13, 1957.

Material and Method

All experiments were carried out on leaves of cherry laurel (*Prunus laurocerasus* Linn.) leaves. A selected leaf was fitted to a water supply and illuminated by an electric lamp, the light intensity at its surface being 1200 ft-c. The oils tested were rosemary and thyme. Two forms of porometer cups, the mouth in both being 7 cm internal diameter, were used for measuring the rate of flow of air through stomata. One of these was the sweep-type previously used and described by the author (2), and the other was a new slightly modified form of it (fig 1). The cups were permanently attached to the leaf and swept, either periodically or throughout the whole period of experiment, by a stream of dry air circulating at a rate of four liters per hour. The air cur-

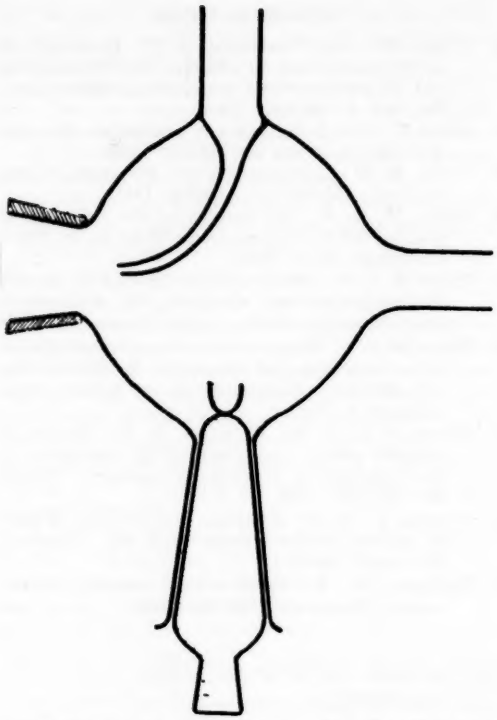


FIG. 1. Porometer cup, designed to permit the application of oil vapors to stomata in still and moving air conditions.

rent was interrupted for short periods while the porometer readings were taken. The time required for the water meniscus in the porometer tube to move 1 cm was measured, under a constant pressure difference of 7.5 cm of water, at 5-minute intervals. In the figures, the 5-minutely porometer readings (as 100/seconds per 1 cm length) are plotted against time.

The temperature was recorded by a thermometer placed very close to the leaf.

RESULTS AND DISCUSSION

The porometer cup used for the first set of experiments was that form used earlier by the author (2). Figure 2 presents the results of three experiments obtained when the circulating dry air was charged, for given periods, with known concentrations of rosemary oil vapor. It can be seen from these experiments that, during the periods of oil treatment, the stomata closed distinctly as indicated by the sudden falling rate of flow through them. On cutting off the oil vapor, stomatal recovery started almost immediately and went on gradually. Recovery was rather complete after the 1st treatment of experiment 2, but only partial elsewhere. Reapplication of the oil vapor in experiments 1 and 2 resulted, again, in similar closing effects.

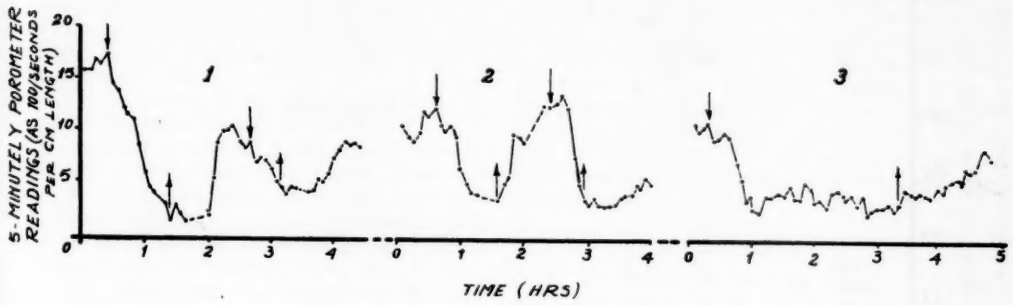
In the following two experiments, shown in figure 3, the procedure was similar to that used before, except that the air stream circulating through the porometer cup was stopped for given periods. In experiment 4, after a period of circulating air during which rosemary vapor was applied and then cut off, the air stream was stopped. During a 2.5-hour period of still air, the stomata opened considerably and gradually with time as shown by the rising stomatal flow rate. This rate was eventually about five times higher than prior to the stoppage of air. When the air stream was recirculated the stomata showed a rapid closing movement and were, within 15 minutes, down to their areas prior to the still-air period.

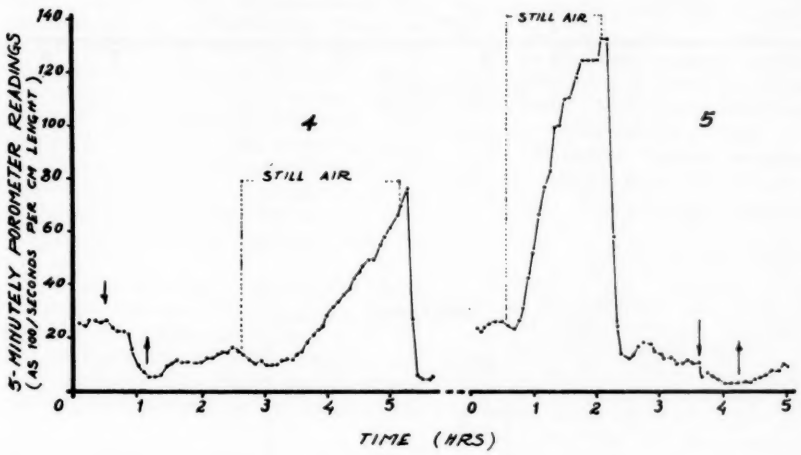
Again, a 1.5-hour period of still air, given in experiment 5 before treatment with rosemary vapor, caused the flow rate through stomata to increase up to about five times its rate just before stoppage of air. When the air current was restarted, the stomatal flow rate decreased within about 15 minutes to below the rate before stoppage, and was down to about one half of the original rate within one hour. The rate seemed to decrease further on applying rosemary vapor, and showed recovery when the vapor was cut off.

The results of experiments 6 to 8 obtained for applied thyme vapor are shown in figure 4. These were generally similar to those for rosemary vapor, the stomatal flow rate being markedly decreased after applying the vapor. But, unlike the results for rosemary, when thyme vapor was cut off, the stomata showed slight or no signs of recovery. Thus, in experiment 7 the air flow through stomata dropped to almost nil by three hours after treatment with thyme vapor, and continued as such even after stoppage of treatment. It would also appear from the results of experiment 8 that, after two successive treatments with thyme vapor in moving air, the stomata failed to show within 40 minutes the usual opening response to still-air conditions.

Two points are noteworthy. First, contrary to Audus and Cheetham's findings, there was a definite closing response of stomata of cherry laurel to applied rosemary and thyme vapors. Second, these stomata opened in still air much wider than in moving air. This suggested the possibility that the failure of Audus and Cheetham to detect any significant closing response was because the stomata through which they measured the resistance to air flow were permanently in still-air conditions, during which time the simultaneous opening movement could possibly mask any expected closing effect. Further experiments were, therefore, needed to test this suggestion.

It should be pointed out that when the above type of porometer cup was used, treatment with an oil vapor could only be achieved by allowing the circulating air to pass over or bubble through the test oil in a container; that is, the oil vapor could be applied only in moving-air conditions. A modified form of porometer cup was therefore designed to give a means of applying the oil vapor in still- as





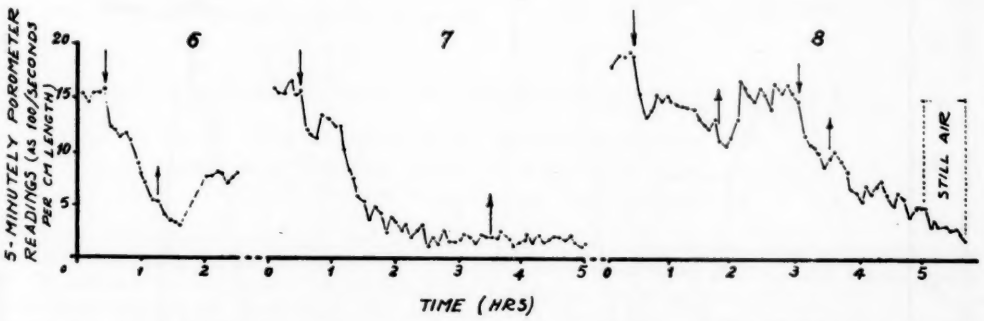


FIG. 2 (*top*). The effect of rosemary oil vapor on the stomata of cherry laurel. Applied vapor concentrations (mg/l) are respectively 17.3 and 16.4 in experiment 1, 16.4 and 16.4 in experiment 2, and 17 5 in experiment 3. N.B. ↓ = Oil vapor on; ↑ = Oil vapor off.

FIG. 3 (*center*). The effects of rosemary oil vapor, and of moving and still air on the stomata of cherry laurel. Vapor concentrations (mg/l) are 13.0 and 15.1 in experiments 4 and 5 respectively.

FIG. 4 (*bottom*). The effect of thyme oil vapor on the stomata of cherry laurel. Vapor concentrations (mg/l) are 10.4 and 11.7 in experiments 6 and 7 respectively, and 20.1 and 12.7 in experiment 8. In experiment 8, the effect of a period of still air is also shown.

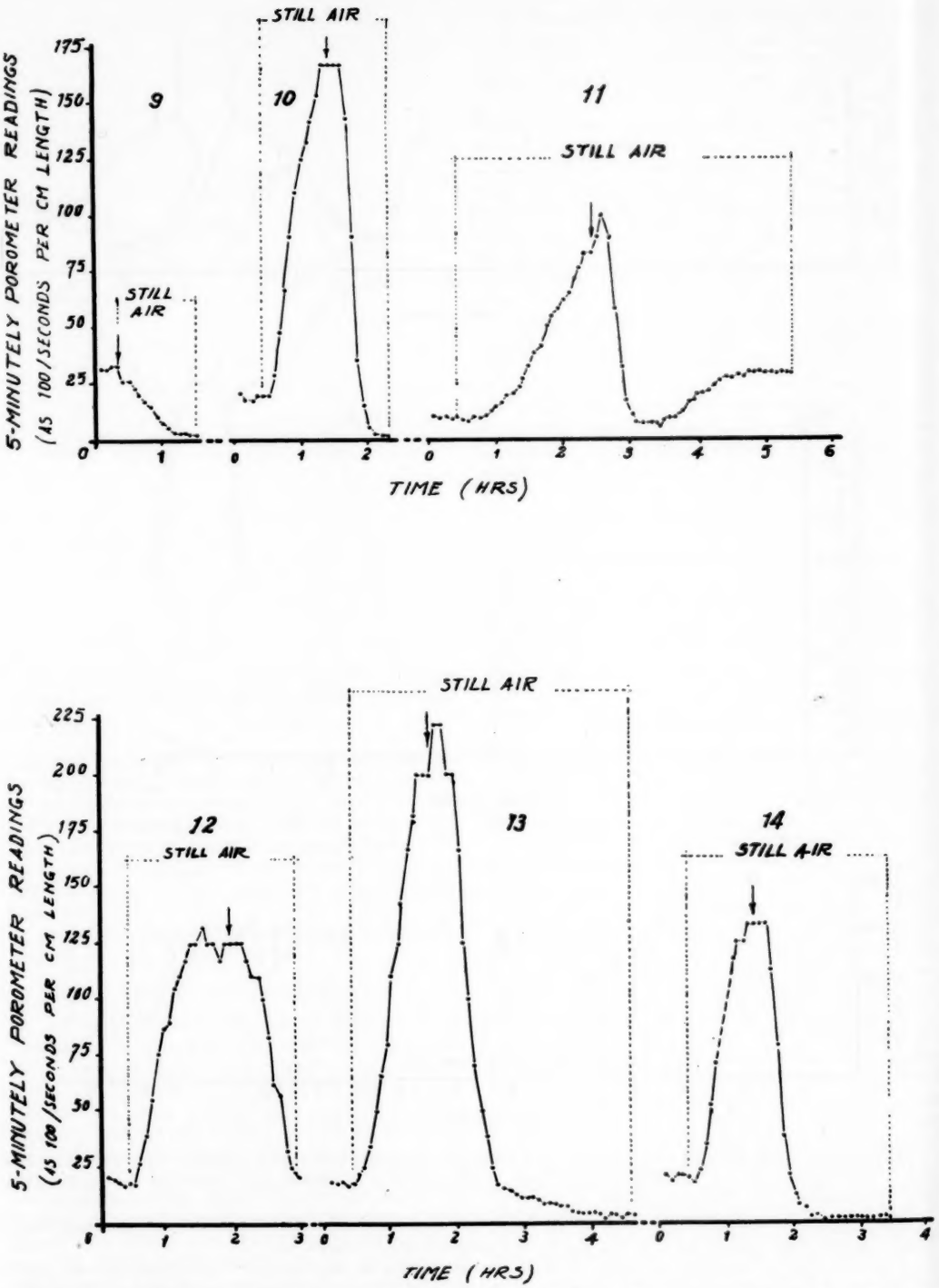


FIG. 5. The effects of rosemary (top, experiments 9 to 11) and thyme (bottom, experiments 12 to 14) vapors applied in still air conditions, on the stomata of cherry laurel.

well as in moving-air conditions. In this modified form, shown in figure 1, opposite to the side tube used as an inlet for a current of air, the cup was provided with an inverted ground stopper with a tiny container sealed onto its tip. Test oil could be placed in this container whenever its vapor was to be applied. The procedure of the following experiments was essentially similar to that used before.

The resulting graphs of three experiments obtained when either rosemary or thyme vapor was applied in still-air conditions are shown in figure 5. In all these experiments the air stream was stopped, after a period of moving air, either at the same or before the time of introducing the oil into the porometer cup. Here, again, the graphs have the same characteristics as those shown before, rising rapidly and considerably on stoppage of circulating air. When rosemary or thyme vapor was applied, the immediate effect on the flow rate through stomata was either a slight temporary increase or no change for about 15 to 25 minutes, followed by a marked decrease. In experiment 11, following the usual closing response to rosemary vapor, stomata showed an appreciable but only partial reopening with time. Only in experiments 13 and 14, portions of the leaf areas treated with thyme vapor showed definite brown spots of injury. In all other experiments of the present work, neither rosemary nor thyme vapor seemed to have any apparent injurious effect on cherry laurel leaves.

The general conclusion to be drawn from all above experiments is that exposure of illuminated leaves of cherry laurel to still or moving air charged with rosemary or thyme vapor brought about a marked stomatal closing response. The closing movement was induced only slightly less readily in still than in moving air.

Although the closing response of stomata of cherry laurel to oil vapors in the present study agrees with the results reported earlier by the author (2) for other oils on other plants, it does not agree with the results of Audus and Cheetham (1) who reported a lack of response by the stomata of the same plant to the vapors of the same oils tested. An explanation for this cannot be obtained from the present work. However, the lack of response, as far as the present experiments indicate, cannot be interpreted, contrary to previous suggestion, on the basis that the opening movement of stomata within a confined space of porometer cup may mask the closing effect of applied vapors.

Summary

1) The effects of applying vapors of rosemary and thyme oils on the stomata of cherry laurel leaves were tested by means of porometer cups.

2) The effects of still and moving air on the stomatal behavior by the same plant were also tested. Stomata opened much wider in still than in moving air.

3) On applying rosemary and thyme vapors to illuminated leaves of cherry laurel, the stomata closed considerably. The closing effect was slightly more rapid in moving than in still air.

4) The gradual opening movement of stomata in still air could not mask their definite closing response to rosemary or thyme vapor treatments.

Literature Cited

1. Audus, L. J. and Cheetham, A. H. Investigations on the significance of ethereal oils in regulating leaf temperatures and transpiration rates. Ann. Bot. N.S. 4: 465–483. 1940.
2. Hafez, M. G. A. Effects of some essential oil vapors on the stomata of Eupatorium and Mentha. Plant Physiol. 33: 177–181. 1958.
3. Heath, O. V. S. and Williams, W. T. Studies in stomatal action. Adequacy of the porometer in the investigation of stomatal aperture. Nature 161: 178–179. 1948.

PHOTOCONTROL OF ANTHOCYANIN SYNTHESIS IN APPLE SKIN[1]

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Red color of apples requires light for its formation and is localized in the areas most exposed to the sun. To enhance the color, some growers expose apples to diffuse sunlight for several days after harvest. The variation in color of apple varieties from green or yellow to various shades of red is indicative of varying capacities for anthocyanin synthesis. The red pigment of the apple skin is cyanidin-3-galactoside, idaein (17).

The literature on the influence of radiation on anthocyanin formation in apple skin was reviewed by Arthur (1). The greatest influence was considered to be between 2900 and 3120 Å, with some response to radiation in the region of 4000 to 6000 Å. Magness (11) found that "dilute applications of ultraviolet light" were effective in promoting the coloration of Jonathan apples. He found also that apples did not color as well under glass as in direct sunlight. Pearce and Streeter (16) considered the maximum action for reddening to be in the region of 3600 to 4500 Å. The action spectrum for anthocyanin synthesis, which expresses the amount of anthocyanin formed for a given energy in various wavelength regions, is reported

[1] Received November 27, 1957.

here for the apple skin at wavelengths greater than 4000 Å. A maximum effectiveness is found in the region of 6500 Å.

Materials and Methods

Early-harvested Jonathan, Rome Beauty and Arkansas apples were held in bushel lots at 0° C sealed in bags made of 0.38-mm polyethylene. Skin on whole apples was used in a few cases, but generally the green areas of the apple skin were removed in strips 15 mm wide with a White Mountain apple peeler. The strips were cut into uniform pieces 7.5 × 7.5 mm or 10 × 15 mm with razor blades mounted in a holder. The pieces, which had some cortical tissue attached, were washed at least three times with about 500 ml of 0.3 M sucrose during a period of about one hour. They were used immediately after washing.

Skin pieces for each experiment were taken from 40 or more apples. They were randomized and 14 pieces were taken for each treatment. The pieces were placed skin side up in 10-cm Petri dishes or in 30-cm Pyrex glass trays containing 0.3 M sucrose and having a filter paper lining in the bottom.

Irradiation was effected with a white fluorescent source of radiant energy giving a maximum illumination of about 1000 foot-candles (ft-c) or with a spectrographic source using a carbon arc source of radiant energy (15). The spectrograph was used as a single-prism instrument and had a dispersion of 13 Å/mm at 7000 Å, 5 Å/mm at 5000 Å, and 2 Å/mm at 4000 Å. Transmission of the prisms limited the region studied to wavelengths longer than 4000 Å. Two slit widths, which subtended 2-cm and 4-cm at the focal position, were used. The 4-cm slit width sacrificed resolution for intensity. The effective widths of the 2-cm slit were 260 Å at 7000 Å, 100 Å at 5000 Å, and 40 Å at 4000 Å. Combination of neutral filters and reflections from front-surfaced mirrors were used to give approximately uniform irradiances between 4100 and 5000 Å. The somewhat greater focal length in the red combined with variations in the sharpness of focus made possible approximately the same intensity from 5800 to 7300 Å. The intensity at 5500 to 5600 Å was 85 % of that at 6100 to 6200 Å. The irradiances were 0.30 milliwatt/cm² for the 2-cm slit and 0.57 milliwatt/cm² for the 4-cm slit in the region of 6100 to 6200 Å. Exposures to the spectrographic source of radiant energy were generally for 12 hours, giving an energy of 13 joules/cm² when the narrow slit was used.

Formation of anthocyanin in apple skin requires a preliminary irradiation period of about 20 hours, the induction period, during which anthocyanin is not formed. After the induction period, anthocyanin synthesis is a linear function of time of irradiation at constant irradiance; for convenience, this is called the linear period.

The action spectrum of the induction period cannot be measured directly. It can be determined only by measuring the amount of anthocyanin formed early in the linear period as a function of the effectiveness of various wavelengths of light in overcoming the radiation requirements of the induction period. The action spectrum of the induction period was measured by irradiating groups of apple skin pieces across the spectrum with the spectrographic source of radiation for 12 hours. The pieces were then irradiated with the fluorescent source of radiation for 20 or 28 hours, after which they were kept 24 hours in darkness.

The action spectrum of the linear period can be measured by determining the effectiveness of various wavelengths of light in promoting anthocyanin formation during the linear period. It was determined by irradiating about 1000 pieces of apple skin with the fluorescent source of radiant energy for 32 or 40 hours. The pieces of apple skin were in the linear period of anthocyanin synthesis after this irradiation. Appreciable red color was evident at this time, and uniformly colored pieces were carefully selected. Groups of 14 selected 7.5 × 7.5 mm pieces were arranged across the spectrum and were irradiated with the spectrographic source of radiation or placed in darkness.

At the conclusion of all irradiation periods, the pieces of apple skin or the whole apples were returned to darkness for 24 hours at 15° C except as noted, to permit synthesis of anthocyanin induced by radiation. The pieces were then removed from the solution and dried by blotting.

The anthocyanin content of the skin was determined on an extract from pieces in each group in 5 ml of 1 % HCl in methanol. When whole apples were used, the skin was removed after the dark incubation, cut into slices, and extracted as just described. The optical density of the extract was generally in the range of 0.1 to 1.0 at 5300 Å. The optical density was also measured at 6500 Å to assess the degree of scattering. The optical density measurements were converted to mole idaein/cm² of apple skin by the use of a molecular-extinction coefficient of 3.43×10^4 at a wavelength of maximum absorption, 5300 Å. The molecular-extinction coefficient was determined on an authentic sample of idaein ($C_{21}H_{21}O_{11} \cdot 2.5\ H_2O$). The copper content of apple skin was determined spectrographically.

Results

The time courses of anthocyanin synthesis in pieces of apple skin and in whole apples are shown in figure 1. Groups of skin pieces or whole apples were irradiated with the fluorescent source of radiant energy for various periods. The three apple varieties examined have an induction period of about 20 hours during which practically no anthocyanin is formed. The induction period is followed by a linear period of anthocyanin formation at constant irradiance. The rate of anthocyanin synthesis in the Rome Beauty apple skin was about 12 % of the rate in the Jonathan apple skin. The linear period of anthocyanin formation in the skin of whole Arkansas apples

is closely parallel to that in pieces of skin, but the duration of the induction period is slightly greater.

The formation of anthocyanin continued in the dark following the irradiation period. Temperature and duration of the dark period following irradiation have considerable influence on the total amount of anthocyanin formed (table I).

The amount of anthocyanin formed as a result of an 84-hour irradiation with the fluorescent source of radiant energy was observed to vary linearly with intensity from an illumination of 270 to 1000 ft-c for skin pieces of Jonathan apple. Apparently a threshold of about 100 ft-c was required before anthocyanin synthesis occurred. The amounts of anthocyanin formed at each intensity expressed as 10^{-8} mole idaein/cm^2 of apple skin in excess of the non-irradiated sample were 120 ft-c, 0.24; 270 ft-c, 1.56; 500 ft-c, 2.88; and 1000 ft-c, 5.88.

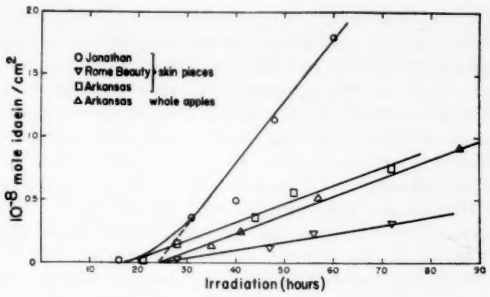
Observations during the formation of anthocyanin indicated that the red coloration of the individual pieces was variable. Groups of skin pieces of Jonathan apples were irradiated with the fluorescent source of radiant energy for 40 hours and then selected for maximum and minimum red color. Portions of the two lots of selected pieces were irradiated for 8, 24, and 48 additional hours with the fluorescent source of radiant energy. Anthocyanin formation in both lots of pieces was linear with time and the rate was 2.9 times as great in the pieces showing the maximum initial red color as in those showing the minimum color.

Injury can promote the formation of anthocyanin in apple skin. Hail and insect damage are frequently recognized by an increase in red color at the site of the injury. Observations on pieces of apple skin showed that anthocyanin formation was evident first at the edge of the slice, where damage occurred in cutting.

The rate of anthocyanin formation in skin pieces held in the dark for 20 hours before irradiation was compared with that of pieces irradiated immediately after preparation. The pieces were floated on 0.3 M sucrose solution containing 1×10^{-3} M pyruvate adjusted to pH 4. The rate of anthocyanin formation was the same for both treatments (fig 2). However, the induction period of the pieces held in darkness for 20 hours before irradiation was reduced about 40 %. A strong odor of apple volatiles developed during the dark incubation. This was not evident in the sample irradiated immediately after preparation.

The copper content and dry weight per unit area of apple skin, scraped to remove some of the cortical tissue, were determined for two apple varieties. The dry weight of the skin was 0.0106 g/cm^2 and the copper content 7 ppm for the Arkansas, and 0.0076 g/cm^2 and 10 ppm for the Jonathan. Examination of cross sections of scraped apple skin revealed that the hypodermal cells, which contain the anthocyanin, did not exceed 15 % of the total volume of the tissue.

Anthocyanin synthesis in pieces of apple skin was found to be unresponsive to the state of the pigment



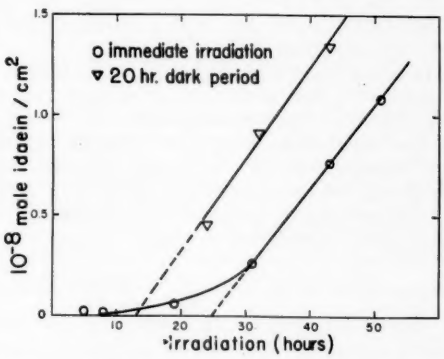


FIG. 1. The dependence of anthocyanin formation in apple skin on the time of exposure under constant irradiance with a fluorescent light source equivalent in photochemical effectiveness to 0.6 milliwatt/cm^2 at 7000 Å. Results are shown for skin pieces of Jonathan, Rome Beauty and Arkansas varieties floating on 0.3 M sucrose and for whole Arkansas apples.

FIG. 2. The dependence of anthocyanin formation in pieces of Arkansas apple skin floating on 0.3 M sucrose solution on the time of exposure at constant irradiance immediately after the preparation of the pieces or after holding them on the sucrose solution in darkness for 20 hours. The fluorescent light source used gave an irradiance equivalent in photochemical effectiveness to 0.6 milliwatt/cm^2 at 7000 Å.

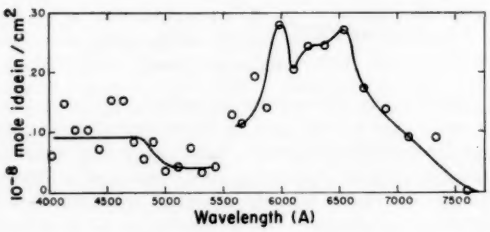
TABLE I

IDAEIN FORMATION IN PIECES OF JONATHAN APPLE SKIN FLOATING ON 0.3 M SUCROSE AS AFFECTED BY DURATION OF DARK PERIOD AND TEMPERATURE FOLLOWING 48 HOURS OF IRRADIATION WITH THE FLUORESCENT SOURCE OF RADIANT ENERGY

DARK PERIOD	IDAEIN FORMED AT INDICATED TEMPERATURE		
	5° C	15° C	25° C
hours	*10^{-9} mole/cm^2*	*10^{-9} mole/cm^2*	*10^{-9} mole/cm^2*
0	...	1.32	...
24	...	2.10	...
48	1.44	2.70	2.16
72	...	2.64	...

for the low-energy photomorphogenic or photoperiodic response. Anthocyanin synthesis in red cabbage seedlings, but not in turnip seedlings, is responsive to the state of the low-energy response pigment (18). Fourteen groups of skin pieces from Jonathan apples were irradiated for 44 hours with the fluorescent source of radiation. One set of 7 of these groups was then exposed for 5 minutes to a far-red source of radiation and the other set to a red source of radiation. The corresponding energies between 6900 and 8000 Å for the far-red source of radiation and between 5800 and 6900 Å for the red source of radiation were about 0.1 joule/cm². The idaein contents of the two sets were far-red, 0.570×10^{-8} mole cm²; and red, 0.574×10^{-8} mole/cm²; with a standard error of 0.004×10^{-8} mole/cm².

The action spectra for anthocyanin synthesis were measured with skin pieces from Jonathan and Arkansas apples. Three measurements of the action spectrum were made during the induction period. Seven measurements of the action spectrum were made during the linear period of anthocyanin formation. Representative results obtained in several action-spectrum experiments are shown in figures 3, 4, and 5. The wavelengths of light most effective in promoting anthocyanin formation were between about 6400 and 6700 Å. A subsidiary action maximum occurred near 6000 Å. In 8 of 10 action-spectrum measurements, anthocyanin formation induced in the region of 6200 Å was lower than that in the region of 6000 Å. In one measurement the values were the same and in another the formation at 6200 Å exceeded that at 6000 Å by 40 %. An estimate of precision calculated for one measurement indicated that the deviation from a smooth curve for the minimum near 6200 Å was significant at the 1 % level, while the deviation for the maximum near 6000 Å was significant at the 5 % level.



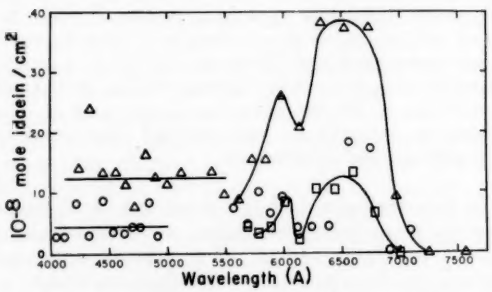


FIG. 3. The action spectrum during the linear phase of anthocyanin formation in pieces of Arkansas apple skin bathed in 0.3 M sucrose. The energy incident on the pieces was about 26 joules/cm² throughout the wavelength region examined.

FIG. 4. The action spectrum during the linear phase of anthocyanin formation in pieces of Jonathan apple skin, floating on 0.3 M sucrose, at an energy of about 26 joules/cm² (upper curve) and about 13 joules/cm² (lower curve) throughout the wavelength region examined. Results from two experiments with the lower energy are shown by circles and squares, respectively.

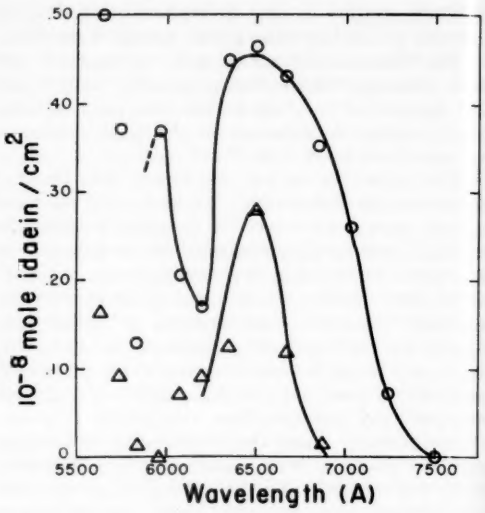


FIG. 5. Action spectrum during the induction phase of anthocyanin formation in pieces of Arkansas apple skin, floating on 0.3 M sucrose, at an energy of about 26 joules/cm² (upper curve) and about 13 joules/cm² (lower curve) throughout the wavelength region examined.

DISCUSSION

The synthesis of anthocyanin in apple skin can be separated into two radiation-dependent phases. The time course of anthocyanin formation has an induction period of about 20 hours in which practically no anthocyanin is formed. The induction period is followed by the linear period, in which anthocyanin formation is a linear function of time at constant irradiance. The induction period is interpreted as the time required to increase the concentration of a substrate to a level permitting the steady state formation of anthocyanin at a rate proportional to intensity.

The nature of the precursors of anthocyanin is suggested in these experiments by partial control of the induction period. The duration of the induction period can be reduced by subjecting apple skin pieces to a dark period before irradiation. In the presence of sucrose a strong odor of apple volatiles develops

during the dark incubation. The relation between the production of volatiles and the reduction of the required induction period is noteworthy. The odor of the apple volatiles was not detected when the apple skin pieces were exposed to the radiation source immediately after preparation. The volatile products of apples are known (9, 12, 13, 20, 21, 23) and consist predominantly of acyl compounds and alcohols. These products provided some suggestions about anthocyanin precursors or alternate products when the photoreaction is withheld. The active fatty-acid metabolism of apple skin, as evidenced by the production of oil and wax (10), also supports the view that acyl groups are precursors of anthocyanin.

Acyl compounds are probably in part the precursors of flavonoid compounds as suggested by Birch (4). Incorporation of acetate into the A ring of flavonoid compounds has been shown by Watkins, Underhill, and Neish (22), Grisebach (7) and Geissman and Swain (5). Grisebach demonstrated by degradation studies that the A ring of cyanidin was derived from a head to tail linkage of three acetate units. The B ring of the flavonoid compounds is derived from the shikimic acid pathway as shown by the work of Watkins, Underhill, and Neish (22) and Geissman and Swain (5). Flavonoid compounds in plants thus arise from a combination of two independent pathways of aromatic biosynthesis.

The action spectrum during the linear period of anthocyanin formation has marked and consistent features. Radiation throughout the visible region of the spectrum is effective in promoting anthocyanin formation, with maximum effectiveness in the red region of the spectrum between 6400 and 6700 Å and a subsidiary maximum near 6000 Å. The action spectrum of anthocyanin formation during the induction period was essentially the same as the action spectrum of the linear phase in the region of 6000 to 7500 Å.

The action spectrum of the linear period of anthocyanin synthesis in apple skin is related to the action spectrum of anthocyanin formation in red cabbage (18), turnip (18), and mustard seedlings (14). The similarities and differences are evident in figure 6. The position of the principal action maximum for the several tissues is in the region of 6500 to 7300 Å. The position of the subsidiary action maximum is near 6000 Å for apple and near 6200 Å for turnip and mustard seedlings. The differences in position of the subsidiary action maximum might not be real but might arise from the proximity of the principal action maximum.

The photoreceptive pigment for anthocyanin synthesis is probably a flavoprotein similar to the acyl coenzyme A (Co A) dehydrogenase enzyme isolated from liver mitochondria by Green, Mii, and Mahler (6). The enzyme is effective in catalyzing the dehydrogenations of acyl Co A compounds such as butyryl Co A. Mahler (12) found that the enzyme contains copper atoms which are effective in single electron transfer. Later work by Steyn-Parve and Beinert

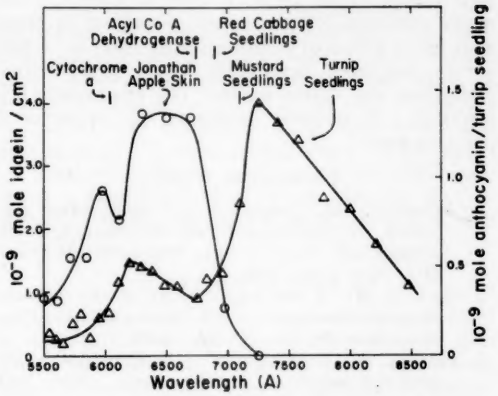


FIG. 6. The action spectrum for anthocyanin formation in pieces of Jonathan apple skin floating on 0.3 M sucrose (radiant energy = 26 joules/cm²) contrasted with that in turnip seedlings (radiant energy = 10 joules/cm²). The wavelengths for action maxima of anthocyanin formation in red cabbage and mustard seedlings and of absorption maxima of cytochrome a and acyl coenzyme A dehydrogenase are shown for comparison.

(19) does not support the presence of copper. The green color of the enzyme may be due to a semiquinoid form of the flavin moiety (3). The oxidized form of the enzyme was shown to activate hydrogen transfer through the functioning of its flavin moiety (12). The absorption spectrum of oxidized butyryl coenzyme A dehydrogenase as determined by Mahler (12) has absorption maxima near 6800 and 4600 Å.

A material balance of anthocyanin synthesis in respect to incident energy and effectively absorbed energy provides pertinent information. The hypodermal tissue, which synthesizes the anthocyanin, consists of 5 or 6 layers of cells and is about 1.0×10^{-2} cm in thickness (2). The maximum concentration of anthocyanin attained during the linear phase after 12 hours of irradiation in the region of 6500 Å is 8×10^{-4} molal in the effective cells. If, during the induction period, an acyl II Co A substrate for anthocyanin synthesis is produced from an acyl I Co A precursor by photoactivation with the same quantum efficiency for both, acyl I Co A would need to reach optimum concentration levels (10^{-3} to 10^{-4} molal) to serve as a substrate for further photoactivation in about 10 hours for the irradiances used. The photoactivation process thus is probably being used at least twice in the course of anthocyanin formation.

SUMMARY

Photocontrol of anthocyanin formation in apple skin was examined. Two distinct radiation-dependent phases were found. The first phase is an induction period of about 20 hours without anthocyanin production. In the second phase anthocyanin formation is a linear function of time of irradiation at constant irradiance. The action spectra for the two phases

were similar in the region of 6000 to 7500 Å. The action spectrum for anthocyanin formation in apple skin has a principal maximum near 6500 Å, a subsidiary maximum near 6000 Å and weak action throughout the visible region. The photoreceptor is probably a flavoprotein similar to acyl coenzyme A dehydrogenase.

Literature Cited

1. Arthur, J. M. Radiation and anthocyanin pigments. In: Biological Effects of Radiation, B. M. Duggar, ed. Vol. II. Pp. 1109–1118. McGraw-Hill, New York 1936.
2. Bain, J. M. A histological study of the development of superficial scald in Granny Smith apples. Jour. Hort. Sci. 31: 234–238. 1956.
3. Beinert, H. Evidence for an intermediate in the oxidation-reduction of flavoproteins. Jour. Biol. Chem. 225: 465–478. 1957.
4. Birch, A. J. Biosynthetic relations of some natural phenolic and enolic compounds. Fortsch. Chem. org. Naturstoffe 14: 186–216. 1957.
5. Geissman, T. A. and Swain, T. Biosynthesis of flavonoid compounds in higher plants. Chem. Ind. 984. 1957
6. Green, D. E., Mii, S. and Mahler, H. R. Studies on the fatty and oxidizing system of animal tissues. III. Butyryl Coenzyme A dehydrogenase. Jour. Biol. Chem. 206: 1–12. 1954.
7. Grisebach, H. Zur Biogenese des Cyanidins. I. Mitt.: Versuche mit Acetat-[1-^{14}C] und Acetat-[2-^{14}C]. Zeits. Naturforsch. 12b: 227–231. 1957.
8. Henze, R. E., Baker, C. E. and Quackenbush, F. W. The chemical composition of apple storage volatiles. I. Acids, alcohols, and esters. Proc. Amer. Soc. Hort. Sci. 61: 237–245. 1953.
9. Huelin, F. E. Volatile products of apples. III. Identification of aldehydes and ketones. Australian Jour. Sci. Res. Ser. B. 5: 328–334. 1952.
10. Huelin, F. V. and Gallop, R. A. Studies in the natural coating of apples. I. Preparation and properties of fractions. Australian Jour. Sci. Res. Ser. B. 4: 526–532. 1951.
11. Magness, J. R. Observations on color development in apples. Proc. Amer. Soc. Hort. Sci. 25: 289–292. 1928.
12. Mahler, H. R. Studies on the fatty acid oxidizing system of animal tissues. IV. The prosthetic group of butyryl Coenzyme A dehydrogenase. Jour. Biol. Chem. 206: 13–26. 1954.
13. Meigh, D. F. Volatile compounds produced by apples. I. Aldehydes and ketones. Jour. Sci. Food Agric. 7: 396–411. 1956.
14. Mohr, H. Der Einfluss Monochromatischer Strahlung auf des Langenwachstum des Hypokotyls und auf die Anthocyanbildung bei Keimlingen von *Sinapis alba* L. [= *Brassica alba* Boiss.]. Planta 49: 389–405. 1957.
15. Parker, M. W., Hendricks, S. B., Borthwick, H. A. and Scully, N. J. Action spectrum for photoperiodic control of floral initiation of short-day plants. Bot. Gaz. 108: 1–26. 1946.
16. Pearce, G. W. and Streeter, L. R. A report on the effect of light on pigment formation in apples. Jour. Biol. Chem. 92: 743–749. 1931.
17. Sando, C. E. Coloring matters of Grimes Golden, Jonathan, Stayman and Winesap apples. Jour. Biol. Chem. 117: 45–56. 1937.
18. Siegelman, H. W. and Hendricks, S. B. Photocontrol of anthocyanin formation in turnip and red cabbage seedlings. Plant Physiol. 32: 393–398. 1957.
19. Steyn-Parve, E. and Beinert, H. Enzyme substrate interaction in a flavoprotein system. Federation Proc. 16: 256. 1957.
20. Thompson, A. R. Volatile products of apples. I. Identification of acids and alcohols. Australian Jour. Sci. Res. Ser. B. 4: 283–292. 1951.
21. Thompson, A. R. and Huelin, F. E. Volatile products of apples. II. Production of volatile esters by Granny Smith apples. Australian Jour. Sci. Res. Ser. B. 4: 544–553. 1951.
22. Watkins, J. E., Underhill, E. W. and Neish, A. C. Biosynthesis of quercetin in buckwheat. Part II. Can. Jour. Biochem. Physiol. 35: 229–237. 1957.
23. White, J. W., Jr. Composition of a volatile fraction of apples. Food Res. 15: 68–78. 1950.

EFFECT OF 6-(SUBSTITUTED)PURINES AND GIBBERELLIN ON THE RATE OF SEED GERMINATION[1]

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Stimulation of lettuce seed germination by some 6-(substituted)aminopurines (3, 5) as well as 6-(substituted)thiopurines (4) has recently been reported. Further, gibberellin has also recently been demonstrated to increase the rate of lettuce seed germination to about the same extent as do the purine derivatives (2). In certain cases, these compounds appear to overcome the light requirements for germination which has been shown to be necessary for certain varieties of seed (1), and in addition, they also exert an effect which augments that of light (5).

In order to study the relationship of the germination effects observed upon lettuce seeds pre-treated with 6-(substituted)purines and gibberellin, the ef-

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fects of combinations of these structurally dissimilar compounds were determined in the present investigation. The results indicate that gibberellin and 6-(substituted)purines are synergistic in increasing the rate of germination of lettuce seed pre-soaked in solutions containing these compounds. Also, the effects of gibberellin and the purine derivatives alone and in combination upon the germination of another variety of lettuce seed and on carpet grass and clover seed have been studied.

Materials and Methods

Lettuce seeds (Early Curled Simpson and Oak Leaf) were pre-soaked for 8 hours, and carpet grass seeds were pre-soaked 12 hours, prior to germination on filter paper wet with the corresponding solutions in Petri dishes; whereas the clover seeds (White Dutch) were not pre-soaked, but placed directly in the Petri dishes on filter paper wet with the corresponding test solutions. The pre-soaking period was carried out in the dark at 25° C, and the seeds were protected from light activation by carrying out subsequent mechanical operations in the presence of blue light (fluorescent light filtered through four layers of du Pont 300 MSC dark blue cellophane). The seeds were germinated either in the dark at 30° C or in the light at 30° C, as indicated in the tables, and 100 or more seeds were used in each assay. When an intermediate time period is reported prior to the terminal count, this count was made in the presence of blue light.

In an effort to determine if there is a specific time sequence of seed activation initiated by these purine compounds during the pre-soaking period, lettuce seeds were treated with a 10 μg/ml solution of 6-benzylaminopurine, and with water separately, for varying time intervals over a combined period of 8 hours. When the seeds were not in contact with the purine solution they were placed in water. Starting with either water or the purine solution, seeds were pre-soaked for one hour in the initial solution, followed by 7 hours in the alternate solution; two hours in the initial solution, followed by 6 hours in the alternate solution, etc., up to 7 hours in the initial solution followed by one hour in the alternate solution, and finally 8 hours in the initial solution. Thus, the seeds were exposed to the active purine solution both prior to and following soaking in water at one hour intervals over a total period of 8 hours. The seeds were then allowed to germinate on filter paper wet with water in covered Petri dishes at 30° C in the dark. It made no significant difference in the eventual germination percentage whether the seeds were initially treated with the purine derivative for 1, 2, or up to 8 hours; or whether the seeds were initially soaked in water and then in the purine solution for any of the 7 time periods, except that the seeds pre-soaked in the purine solution had a higher germination percentage (about 10 % higher) than did corresponding seeds initially pre-soaked in water and then placed in the purine solution.

The synergism was shown experimentally by pre-soaking the seeds in a solution containing the indicated concentration of purine and gibberellin [2] for 8 hours, followed by germination on filter paper in covered Petri dishes at 30° C in the dark. A similar experiment was conducted using indoleacetic acid with some of the more active purine derivatives in an effort to determine whether or not auxin possessed a synergistic effect in combination with 6-(substiuted)purine compounds on stimulation of seed germination.

Results and Discussion

The stimulatory effect on germination produced by pre-soaking seeds in 6-(substituted)purine solutions is not limited to lettuce seed alone, but has been found to be applicable to other varieties of seed as well. An appreciable increase in rate of germination was observed in the case of carpet grass after pre-soaking in several of the more active purine solutions as indicated in table I; however, the concentration required for activation of this seed is somewhat higher than that required for activation of the lettuce seed. Pre-treatment of carpet grass seeds with 30 μg/ml of 6-pentylamino-, 10 μg/ml of 6-benzylamino- or 6-(2-thenyl)aminopurine for 12 hours, followed by germination in the dark for 7 days, gave 56 %, 52 % and 41 % germination, respectively, as compared to a water control of 12 %. These stimulatory effects are also observed even when the seeds are allowed to germinate in the light at 30° C, wherein the corresponding values for the compounds are 71 %, 70 % and 70 % respectively, as compared to a water control of 35 %. These results are comparable in kind to those observed with White Dutch clover as indicated in table I as well as those previously reported for Early Curled Simpson lettuce seed (5). However, whereas the carpet grass and lettuce seeds after pre-soaking in water showed an appreciable light stimulation when allowed to germinate at 30° C, the clover seed did not. Thus, clover seeds pre-soaked in water gave 33 % germination under the usual test conditions in the dark after 96 hours, and seeds in the light were only 39 % germinated in the same time interval. In contrast, the seeds pre-treated with the more active purine compounds had a significantly higher germination percentage under both sets of experimental conditions; for example, pre-soaking in a 3 μg/ml of 6-benzylaminopurine gave values of 74 % and 71 % after germination in the dark and light at 30° C, respectively. Thus, these compounds appear to activate some system(s) governing germination other than those activated by light.

In an attempt to determine if there is an auxin involvement in the purine induced germination of seed, 10 μg/ml solutions of several of the amino and

[2] The gibberellin was obtained through Dr. J. L. Liverman of Texas A. and M. College and was a commercial sample issued by Merck and Co. under the name "Gibrel."

TABLE I

STIMULATION OF SEED GERMINATION BY SOME 6-(SUBSTITUTED)PURINES *

COMPOUND †† 6-(SUBSTITUTED)-PURINE	CONCENTRATION, μG/ML	CARPET GRASS **		WHITE DUTCH CLOVER †			
		PERCENT OF SEED GERMINATED AFTER: (DAYS)					
		7 (Lt)	7 (Dk)	2 (Lt)	2 (Dk)	4 (Lt)	4 (Dk)
Water blank control	..	35	10	22	12	39	33
Pentylamino-	1	33	18	58	17	64	22
	3	49	21	58	42	76	45
	10	59	40	71	30	70	52
	30	71	56	37	19	60	54
Hexylamino-	1	39	16	44	27	63	33
	3	47	14	61	37	69	52
	10	60	28	64	33	76	50
	30	61	41	25	49	57	64
Benzylamino-	0.3	39	25	61	45	77	64
	1	51	31	65	60	72	68
	3	55	41	65	60	71	74
	10	70	52	51	24	62	33
Kinetin	0.3	39	14	20	14	29	24
	1	49	18	49	52	73	63
	3	50	24	63	36	70	57
	10	64	39	14	12	30	25
Thenylamino-	0.3	36	11	61	48	68	58
	1	41	16	59	56	69	70
	3	60	32	57	43	67	60
	10	67	41	63	49	61	64
3-Pyridylmethyl-amino-	1	39	18	30	46	37	63
	3	45	24	46	48	65	53
	10	55	24	62	52	66	59
	30	68	39	60	60	65	68

* 100 or more seeds used in each assay.

** Pre-soaked in the indicated solution for 12 hours, allowed to germinate in the light (Lt) or dark (Dk) at 30° C.

† Seeds not pre-soaked; placed directly on filter paper containing the indicated solution and allowed to germinate in the light (Lt) or dark (Dk) at 30° C.

†† Several thiopurine analogues were tested and found to be appreciably less active than the corresponding amino compounds; however, some stimulation of germination was observed.

thiopurine derivatives were supplemented with 1 and 10 μg/ml of indoleacetic acid, and the resulting solutions were used for pre-soaking lettuce seeds. The results obtained after germination in the dark at 30° C are presented in table II. No appreciable increased rate of germination was observed; however, in many instances the higher concentrations of auxin appeared to inhibit the action of the purine derivative in stimulating germination of lettuce seeds. Since the active concentration of auxins are sometimes quite specific, the effect of varying amounts of indoleacetic acid in the presence of 6-benzylaminopurine on lettuce seed germination was studied over a much wider range of concentrations. The amount of auxin used for pre-soaking lettuce seed was varied in ten-fold increments from 10^{-6} to 1.0 μg/ml in the presence of 0.01, 0.1 and 1.0 μg/ml of the purine derivative. The auxin alone produced no stimulatory effects, and the combination of the two compounds at all of these concentrations gave values which were comparable to those for seeds pre-soaked in the purine solution alone.

Since gibberellin (2), as well as purine derivatives, have been observed to stimulate seed germination, it was decided to examine the effect of pre-soaking seeds in a combination of these two structurally dissimilar compounds. Such a combination proved to be synergistic in stimulating seed germination as indicated in table III. Pre-soaking Early Curled Simpson lettuce seeds in gibberellin solutions at 30 and 100 μg/ml gave 11 and 15 % germination, respectively, while pre-soaking the seed in 1 and 10 μg/ml of 6-benzylaminopurine resulted in 4 and 23 % germination, respectively. A combination of gibberellin and the purine derivatives yielded, in every case, a marked synergistic stimulation of rate of lettuce seed germination. For example, a mixture of 1 μg/ml of 6-benzylaminopurine and 30 μg/ml of gibberellin gave 71 % germination. The thiopurine analogs were less effective than the corresponding amino-

TABLE II

EFFECT OF 6-(SUBSTITUTED)PURINES AND AUXIN ON THE GERMINATION OF LETTUCE SEED *

6-(SUBSTITUTED)-PURINE, 10 μG/ML	INDOLEACETIC ACID CONC, μG/ML	PERCENT OF SEED GERMINATED AFTER: (HOURS)	
		48	96
None	0	2	19
	1	2	17
	10	0	3
Phenylethylamino-	0	74	81
	1	58	74
	10	27	51
Phenylethylthio-	0	22	60
	1	27	65
	10	7	55
Butylamino-	0	83	92
	1	63	81
	10	39	63
Butylthio-	0	9	40
	1	14	39
	10	4	31
Heptylamino-	0	66	76
	1	44	71
	10	19	66
Heptylthio-	0	3	15
	1	4	19
	10	0	16
2-Thenylamino-	0	68	83
	1	54	73
	10	34	57

* In each assay 100 or more Early Curled Simpson lettuce seeds were pre-soaked for 8 hours in the dark at 25° C and germinated on wet filter paper in the dark at 30° C.

purines; however, a stimulation was observed when the seeds were pre-soaked in a mixture of the thiopurine analogs and gibberellin over that of those seeds soaked in either of the compounds alone. A similar synergistic stimulation of germination was also observed with 6-benzylaminopurine and gibberellin using another variety of lettuce seed as indicated in table IV.

The observed stimulation of germination of carpet grass and clover seeds after pre-soaking in the purine solutions was extended to an examination for a possible synergistic effect using gibberellin and the 6-(substituted)purine derivatives with these seeds. Some increase in stimulation of germination was observed with carpet grass seeds pre-soaked in mixtures of gibberellin and 6-benzylaminopurine, as indicated in table IV. In contrast, no appreciable effect was noted, under the test conditions used, for a stimulation of germination of clover seed over that of the stimulatory effect of the purine derivatives alone.

The structural feature of the purine derivative initiating this activity does not appear to be solely the purine nucleus, since adenine, at similar concentrations, gave no stimulatory effects either alone or in the presence of gibberellin. The fact that both the thio- and amino- analogs affect the rate of germination of lettuce seed indicates that structural specificity is not very exacting. However, it appears that the purine structure itself may be necessary to produce an active analog since the corresponding 4-(substituted)-pyrazolo(3,4-d)pyridine derivatives of some of the more active purine analogs were essentially inactive in this type assay system (6).

The synergistic effect of the purine derivatives and gibberellin indicate that these substances differ in their mode of action but are probably closely related in their functions. It appears likely that their effects are exerted in sequence in certain plant systems.

Table III

Synergistic Germination Effects of Gibberellin and Some 6-(Substituted)purines *

6-(Substituted)-purine	Conc, μg/ml	Gibberellin, μg/ml		
		0	30	100
		Percent of seed germinated after 24 hours **		
None	..	1	11	15
6-*n*-Hexylamino-	1	8	69	54
	10	17	80	82
6-*n*-Hexylthio-	1	1	21	27
	10	12	40	53
6-Benzylamino-	1	9	71	69
	10	23	85	93
6-Benzylthio-	1	0	24	21
	10	4	39	56
Kinetin	1	15	69	89
	10	29	85	81
Adenine	1	2	6	16
	10	1	6	7

* Early Curled Simpson lettuce seeds were pre-soaked for 8 hours in the indicated solutions, and allowed to germinate in the dark at 30° C.

** 100 or more seeds were used in each test.

Table IV

Activity of Gibberellin and 6-Benzylaminopurine on the Rate of Germination of Some Seed

6-Benzylamino-purine conc, μg/ml	Time interval of germination, days	Gibberellin concentration, μg/ml			
		0	30	100	300
		Percent of seed germinated in time interval			
Carpet grass *					
0	5	13	13	18	18
0	7	18	19	24	21
1	5	15	18	23	27
1	7	21	26	34	34
10	5	27	41	47	51
10	7	37	45	50	57
White Dutch clover **					
0	1	7	5	12	8
0	2	20	14	23	22
1	1	36	29	36	34
1	2	61	55	56	55
10	1	35	36	31	36
10	2	57	57	40	55
Oak Leaf lettuce ***					
0	1	3	15	41	36
1	1	36	63	77	88
10	1	58	81	84	89

* Pre-soaked for 12 hours in the indicated solution in the dark; germinated in the dark at 30° C.

** Seeds placed directly on filter paper wet with the indicated solution and allowed to germinate in the dark at 30° C.

*** Seeds pre-soaked for 8 hours in the indicated solutions in the dark; germinated in the dark at 30° C.

Summary

The previously observed stimulation of the rate of seed germination by pre-soaking lettuce seeds in 6-(substituted)purine solutions has been extended to a comparable stimulation effect using carpet grass and clover seeds. Further, a combination of gibberellin and 6-(substituted)purines has been found to act synergistically in stimulating the rate of germination of lettuce and carpet grass seeds under certain experimental conditions. These results indicate different modes of action of gibberellin and 6-(substituted)purines and suggest that these substances are closely related in their biochemical functions, which are perhaps in sequence.

Literature Cited

1. Borthwick, H. A., Hendricks, S. B., Parker, M. W., Toole, E. H. and Toole, Vivian K. A reversible photoreaction controlling seed germination. Proc. Nat. Acad. Sci., U.S. 38: 662–666. 1952.
2. Kahn, A. and Goss, J. A. Effect of gibberellin on

germination of lettuce seed. Science 125: 645–646. 1957.
3. MILLER, C. O. Similarity of some kinetin and red light effects. Plant Physiol. 31: 318–319. 1956.
4. SKINNER, C. G., CLAYBROOK, J. R., TALBERT, F. and SHIVE, W. Stimulation of seed germination by 6-(substituted)thiopurines. Arch. Biochem. Biophys. 65: 567–569. 1956.
5. SKINNER, C. G., CLAYBROOK, J. R., TALBERT, F. and SHIVE, W. Effect of 6-(substituted)thio- and amino-purines on germination of lettuce seed. Plant Physiol. 32: 117–120. 1957.
6. SKINNER, C. G. and SHIVE, W. Effect of some isomeric purine analogues on germination of lettuce seed. Plant Physiol. 32: 500–501. 1957.

THE FIXATION OF $C^{14}O_2$ INTO TARTARIC AND MALIC ACIDS OF EXCISED GRAPE LEAVES[1,2]

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Tartaric acid is the major organic acid in the leaves and fruits of the grape (*Vitis vinifera*) (2, 3, 9, 10), and together with malic acid, constitute 80 to 90 % of the total organic acid fraction. Although the data are not conclusive, there is evidence that both tartaric and malic acids are synthesized only in the leaves, and are translocated to the ripening fruits during the late summer (9). The malic acid content decreases rapidly both in leaves and in fruits under certain conditions, but the tartaric acid content remains relatively constant. Leaves picked in the early morning or night showed no significant change in the amounts of malic or tartaric acids, suggesting that there is little or no diurnal variation in these organic acids (9).

No specific enzymatic reactions involving (+)-tartaric acid are known in higher plants, although bacteria and fungi can oxidize or dehydrate all three isomers (13, 15, 16, 20, 21, 28). Extracts from higher plants and animals, on the other hand, can oxidize (−)- and meso-tartaric acid, and there is preliminary evidence that seed tissues of higher plants can also dehydrate (−)- and meso-tartaric acid forming oxaloacetic acid (23, Stafford, unpublished data). The formation of meso-tartaric acid has been demonstrated in several fungi, bacteria, and a yeast (15).

The present study was undertaken to gain information concerning the metabolic origin of tartaric acid, using in vivo techniques and $C^{14}O_2$ as a source of label. The results indicate that tartaric acid is formed in a secondary process from the primary carbohydrate products of photosynthesis. Organic acids formed by CO_2 fixation, such as malic acid, do not appear to be likely precursors.

MATERIALS AND METHODS

The 2nd or 3rd leaves from the growing tips of the fruiting vines of the Mission variety of grape were picked at about 9:00 AM, and the petioles were immediately recut under water. In each experiment, three leaves were used. These were placed in a beaker of water under a micro bell jar (325 ml capacity) fitted with tubulation and a ground glass bottomed plate. A vial containing dry $BaC^{14}O_3$ was connected to the tubulation with a short piece of rubber tubing and $C^{14}O_2$ was released by injecting a few milliliters of 50 % lactic acid into the vial via the rubber tube. The jars were then either placed in the dark (experiment 2) or in the light (experiments 1, 3, and 4) under two 20-watt daylight fluorescent tubes at a distance of about 10 cm at room temperature (about 25° C). After three hours in the light, the remaining $C^{14}O_2$ in experiment 4 was removed by aspirating through a *N* NaOH trap and was replaced with $C^{14}O_2$-free air. This bell jar was then placed in the dark for the remaining 21 hours.

At the end of the experimental period, all bell jars were aspirated, the leaves weighed, plunged into boiling alcohol for two minutes and frozen in a dry ice-alcohol mixture prior to homogenization (in a Virtis homogenizer). After centrifugation of the homogenate and decantation of the supernatant, the residue was resuspended in water and decationized with Dowex 50 (H^+ form) batchwise to insure the solubilization of any remaining potassium acid tartrate. These decationized washings were added to the original supernatant, the combined extract was concentrated in vacuo at 40° C to remove the alcohol, and was centrifuged at high speed. Finally, the supernatant was filtered through a Dowex 50 column (H^+) (1.5 × 7 cm) to remove any remaining cations. The effluent, which was composed primarily of sugars and organic acids was added to a Dowex 1 column (formate, 200 to 400 mesh, 1.2 × 8 cm). After washing the column with 2 or 3 column volumes of distilled water, a 3.0 M formic acid gradient was started (17) and the eluted acids were collected in 100 successive 4-ml fractions. A 0.1- to 0.2-ml aliquot from each tube was used to estimate the activity of the fraction. This was accomplished by plating the aliquot on a steel planchet and measuring the activity under a thin-window, gas flow counter in the Geiger

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region. The remainder of each fraction was evaporated to dryness with a current of air at 47° C (17) to remove the formic acid. The non-volatile acid residues of each fraction were then taken up in boiled distilled water and titrated with 0.01 *N* NaOH. From the information obtained by titration, chromatography, and measurement of the radioactivity, the eluate from the Dowex 1 column was divided into four major fractions: a mixture referred to as pre-malic, a malic, a tartaric, and finally, a citric fraction. The combined fractions from each peak were decationized by passage through a small column of Dowex 50 (H^+) and concentrated to a 5-ml volume. Small aliquots were titrated with 0.01 *N* NaOH using a micropipette and were analyzed for organic acids by paper chromatography (17). The malic and tartaric acids were diluted with unlabeled material and then crystallized to constant specific activity. Malic acid was crystallized three times from an ethyl acetate-petroleum ether mixture. Tartaric acid was crystallized four times as the potassium acid salt from water.

Total wet combustions of malic and tartaric acids were accomplished using a Van Slyke-Folch reagent (25). In addition, tartaric acid was totally converted to CO_2 by the lead tetraacetate-potassium acetate procedure of Perlin (18).

Partial degradation of tartaric acid was effected by oxidation with sodium metaperiodate (22) which released the two terminal carboxyl carbons together as CO_2. C-2 and C-3 of tartaric acid which remained in solution as formic acid were then further oxidized with lead tetraacetate to CO_2 (18).

Malic acid was partially degraded to CO_2 (C-4 or the beta-carboxyl carbon) and lactic acid by a lyophilized cell suspension of malic-adapted *Lactobacillus arabinosus* (5). The lactic acid was further degraded chemically (11) to acetic acid (C-2 and C-3) and CO_2 (C-1). Acetic acid was converted to CO_2 by wet combustion as a rough check on the activity of C-2 and C-3 as determined by difference from the total wet combustion and the partial degradation.

The activity of each CO_2 sample was counted directly in the gas phase using methane as the counting gas by the method of Bernstein and Ballentine (4, 26). Results are reported in cpm per mg of carbon and correspond to about 80 % of the C^{14} disintegrations.

Experimental Results

$C^{14}O_2$ Fixation: Analyses of the amounts of the three major organic acid fractions in excised grape leaves after different experimental treatments are shown in table I. The amount of tartaric acid per gram fresh weight of leaf was relatively constant for all four experiments, while the values for malic acid tended to vary with the treatment, the lowest amounts being found in leaves subjected to a dark treatment. It is doubtful whether the age difference of the plant used in experiment 1 had any effect since leaves of a similar developmental stage were always chosen. The ratio of tartaric to malic content varies from 3 to 6 (2). Paper chromatography indicated that the tartaric and malic fractions were not contaminated with any significant amounts of other organic acids. The "citric" acid fraction was a complex, yielding three to four spots upon chromatography one of which was tartaric acid due to the tailing of this acid in the formic acid gradient.

The malic acid peak was characterized by the coincidence of its acidity and its radioactivity. The tartaric acid peak was characterized by its acidity and by use of the ammonium metavanadate spot test (6). The "citric" acid peak contained too little acid to be detectable by titration. It was characterized initially on the basis of activity and subsequently the combined fractions were concentrated to a small volume to permit a more complete evaluation. Paper chromatography revealed that most of the acidity and activity was associated with a spot at the R_f characteristic of citric acid.

Table II summarizes the data on the activity found in the various fractions of each experiment. The method employed in counting the thin solid-sample aliquots had an efficiency approaching 50 % of the total C^{14} disintegrations, thus permitting a very rough comparison of the total activity in a given fraction with the $C^{14}O_2$ administered. The activities in table II are corrected for background only. The total number of cpm found in the soluble extract is given at the top of each column. The percent recovered at each succeeding step of the separation is also recorded.

As might be expected, experiment 3, involving the longest light exposure in $C^{14}O_2$ resulted in the greatest incorporation of C^{14} into soluble constituents,

Table I

Analyses of Major Organic Acids after Separation on Dowex 1 (Formate)

Expt no.	Treatment of excised leaves	Date leaves picked	$C^{14}O_2$ used	Final wet weight of leaves	Tartaric acid	Malic acid	Acids in "citric" peak
			μC	*grams*	*μequiv/g wet wt of leaves*		
1	$C^{14}O_2$—3 hrs in light	6-21-57	17.8	2.0	193	66	...
2	$C^{14}O_2$—24 hrs in dark	7-16-57	28.3	2.3	204	37	14
3	$C^{14}O_2$—24 hrs in light	7-16-57	31.1	1.8	205	57	7
4	$C^{14}O_2$—3 hrs in light + CO_2—21 hrs in dark	7-16-57	26.7	1.7	182	45	4

TABLE II

SUMMARY OF ACTIVITY DURING FRACTIONATION AS ESTIMATED FROM SOLID SAMPLE DISC COUNTS

EXPERIMENTAL TREATMENT	EXPT 1	EXPT 2	EXPT 3	EXPT 4
	$C^{14}O_2$—3 HRS LIGHT	$C^{14}O_2$—24 HRS DARK	$C^{14}O_2$—24 HRS LIGHT	$C^{14}O_2$—3 HRS DARK + CO_2—21 HRS DARK
Activity of original supernatant (sugars, amino acids, organic acids)	4,250 000 cpm 100 %	541,000 cpm 100 %	17,000,000 cpm 100 %	9,600,000 cpm 100 %
Activity in Dowex 50 effluent (sugars, organic acids)	...	89	95	94
Activity in Dowex 1 effluent (sugars)	53	5	62	77
Activity bound by Dowex 1 column	47	84	33	17
Activity eluted from Dowex 1 by 3 *N* formic acid	31	68	15	17
Further activity eluted from Dowex 1 by 6 *N* formic acid	2	0.5	0	0
Activity remaining on Dowex 1 after elution with 6 *N* formic acid *	14	16	18	0
Distribution of activity in fraction eluted from Dowex 1 with 3 *N* formic acid				
Total counts, cpm	1,300,000	370,000	2,500,000	1,600,000
Pre-malic	12 %	1 %	12 %	17 %
Malic peak	77	91	82	76
Tartaric peak	(0.02)	(0.00)	(0.01)	(0.08)
"Citric" peak	7	6	4	5

* Calculated by difference between total activity bound on Dowex 1 and activity eluted by 3 *N* and 6 *N* formic acid.

notably the sugars. Conversely, very little $C^{14}O_2$ was converted to sugar in the dark (experiment 2) and a major share of the label occurred in the organic acid fraction. The acids of greatest concern to this investigation appeared in the 3 *N* formic acid eluate from Dowex 1. Information concerning the total activity and the percent distribution of label in this eluate is given in the lower portion of table II. Malic acid accounted for the major portion of the activity in all experiments (77 to 91 %). The pre-malic fraction contained appreciable activity in all light experiments. In experiment 1, this fraction consisted of at least four well characterized radioactive peaks. With longer periods of light or dark, only one peak could be detected. Negligible activity was found in the tartaric acid peak as determined by plating small aliquots for a thin solid-sample count. An estimate of the C^{14} content of tartaric acid could, however, be made after isolation and recrystallization by means of the more sensitive gas counting procedure. These values are recorded in brackets in table II.

The specific activities of the malic and tartaric acids isolated from the leaves together with the results of partial degradation of these compounds are summarized in table III. The various methods of oxidation employed are indicated in footnotes. No detectable amount of radioactivity was found in tartaric acid after treatment with $C^{14}O_2$ in complete darkness. The gas-phase counting procedure is extremely sensitive and in this particular case the size of sample taken for combustion was such that a minimum of three C^{14} disintegrations per minute above background would have been detected. C^{14} was incorporated into tartaric acid in the light. Fixation of label was best when three hours of light was followed by 21 hours of darkness despite the absence of $C^{14}O_2$. In fact, a continuous treatment with light appeared to decrease the final amount of C^{14} incorporated.

Degradation studies indicated that the two internal carbons of tartaric acid became labeled more rapidly than the external ones in the shortest light treatment (experiment 1) but that the label became randomized with longer treatment (experiments 3 and 4). The oxidation technique did not differentiate between the two internal (or external) carbons so that it is not known whether only one or both of these carbons became labeled.[4]

Malic acid was radioactive in all experiments, but the highest specific activity was found after three hours of light followed by 21 hours of darkness or after 24 hours of light. The degradation of malic acid, which was effected with a lyophilized suspension of malic-adapted *Lactobacillus arabinosus*, permitted separate determination of the activities of C-1 and C-4. Only the sum of the activities of C-1 and C-4

[4] An experimental approach to the stepwise degradation of (+)-tartaric acid (or the other isomers) is provided in the work of Shilo and Stanier (20, 21). These workers have demonstrated the conversion of all three isomers of tartaric acid to oxalacetic acid by adaptive bacterial enzymes. In the presence of malic dehydrogenase and diphosphopyridine nucleotide, tartaric acid could be converted to L-malic acid. The stepwise degradation of the latter compound is known (5).

are reported in table III. The present study indicated that label appeared first in the carboxyl carbons, equally distributed, as has been reported in other tissues (1, 27). In the light, there was a tendency toward randomization of C^{14} throughout the molecule, especially after 24 hours of light or three hours of light followed by 21 hours of darkness.

ENZYME STUDIES: Grape leaf enzyme preparations were examined for some of their dehydrogenase or reductase activities. Preparations were active only if the excised leaves were vacuum infiltrated with a 1 % ammonia solution (7) before lyophilization and subsequent treatment with acetone and ammonium sulfate as described in former studies (23). These grape leaf extracts (table IV) contained the following activities: malic dehydrogenase, tartaric dehydrogenase, diketosuccinate reductase, and ketomalonate reductase. Only the (−) and meso isomers of tartaric acid were active as substrates with tartaric dehydrogenase. No tartaric dehydrase activity could be demonstrated with any of the three isomers, an observation previously made on other leaf preparations and in contrast to seed extracts which do contain this activity (Stafford, unpublished data).

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN MALIC AND TARTARIC ACIDS FROM GRAPE LEAVES LABELED WITH $C^{14}O_2$

CARBON FRAGMENT	MALIC ACID		TARTARIC ACID		
	SPECIFIC ACTIVITY	PERCENT OF TOTAL ACTIVITY		SPECIFIC ACTIVITY	PERCENT OF TOTAL ACTIVITY
	cpm/mg C			*cpm/mg C*	
Expt 1					
$C_{1+2+3+4}$	760,000 *	100	Run 1:	100 †	100
			2:	112 ***	100
C_{1+4}		85 **	1:		24 ‡
			2:		25 ‡
C_{2+3}		15 ***	1:		76 ***
			2:		75 †
Expt 2					
$C_{1+2+3+4}$	334,000 *	100		0 *	
C_{1+4}		100 **			
C_{2+3}		0 ***			
Expt 3					
$C_{1+2+3+4}$	1,610,000 *	100		29 *	100
C_{1+4}		65 **			55 ‡
C_{2+3}		35 ***			45 ***
Expt 4					
$C_{1+2+3+4}$	1,740,000 *	100	Run 1:	306 *	100
			2:	330 ***	100
C_{1+4}		61 **	1:		48 ‡
			2:		50 ‡
C_{2+3}		39 ***	1:		52 ***
			2:		50 †

* Van Slyke-Folch wet combustion.
** Stepwise degradation with *L. arabinosus*.
*** Calculated by sum or difference of degraded fractions.
† Oxidation with lead tetraacetate-potassium acetate.
‡ Oxidation with sodium metaperiodate.

TABLE IV

ENZYMATIC ACTIVITIES FOUND IN EXTRACTS FROM YOUNG GRAPE LEAVES *

ENZYME	ACTIVITY	
	PREP A	PREP A-P
	Δ O.D./min/mg protein	
Malic dehydrogenase (as oxalacetic reductase)	136	210
Meso-Tartaric dehydrogenase	0.023	0.070
(−)-Tartaric dehydrogenase	Not detectable	0.019
Ketomalonic reductase	3.0	.....
Diketosuccinic reductase	3.2	.....

The assays were run at 340 mμ using a 3.0-ml volume (1.0-cm cuvette) containing: 0.2 ml TRIS buffer, 0.5 M (pH 7.4 for DPNH oxidation, pH 9.0 for DPN reduction), 0.2 mg DPN or DPNH, 0.07 to 7.0 mg protein (leaf extract), and 100 to 200 micromole of appropriate substrate. Preparation A was the enzyme fraction obtained between 0 and 400 g ammonium sulfate per liter of soluble extract (21). Preparation A-P was the fraction obtained between 100 and 400 g ammonium sulfate per liter from preparation A.

* Thompson seedless grape plants. The 2nd and 3rd leaves were cut from actively growing vines on the morning of July 26, 1957, at Davis, California. Infiltration of the leaves with 1 % ammonia was effected within 1 hour of picking.

DISCUSSION

The universality of tartaric acid in plants is questionable (29, 30) but reports of the presence of this acid in plants are constantly appearing (19). Grapes, at any rate, are unusual in that tartaric acid frequently replaces malic acid as the principal organic acid. The grape leaf does, however, incorporate a major portion of the CO_2 fixed by organic acids, into malic acid, just as in the case of other plants (27).

Although the optical rotation of the tartaric acid isolated was not checked, it did give a positive metavanadate test indicating the presence of either one of the optically active isomers. Presumably it was all (+) isomer, but this point needs to be examined more carefully. The only known enzymes in higher plants which metabolize tartaric acid do not use the (+) isomer which is found naturally in such large quantities in the grape.

Not only is tartaric acid rather slowly synthesized, but, once it is formed, it is either completely stable in the plant or is metabolized only very sluggishly. These observations are simlar to those reported by Vickery (29, 30) who infiltrated tobacco leaves with (+)-tartaric acid. The fact that the specific activity of tartaric acid in the grape leaf was lower after 24 hours of light than after only three hours suggests that there might be a slow disappearance. More experiments would be necessary to check this point. Malic acid, on the other hand, is much more actively formed, the label appearing initially in the carboxyl carbons. Furthermore, malic acid becomes labeled

in the dark, presumably by exchange reactions as well as by dark CO_2 fixation, while light appears to be required for the incorporation of any significant amount of label into tartaric acid.

All of the above points; i.e., the enzymatic data, the relative sluggishness of the tartaric system in contrast to that of malic acid, and the requirement of light for the synthesis of tartaric acid from CO_2 appear to rule out malic acid or perhaps any Krebs cycle acid as the precursor of (+)-tartaric acid in grape leaves. Instead, these points plus the initial labeling of the internal carbons of tartaric acid instead of the carboxyl carbons appear to suggest that a sugar is the more likely precursor. Such a view is consistent with the results obtained when a grape leaf was administered L-ascorbic acid-6-C^{14} (14). In this latter experiment, tartaric acid appeared to receive C^{14} by a secondary process, the sugars being labeled initially. Just how the conversion of a sugar such as glucose to tartaric acid might occur is still a matter of conjecture. Possible intermediates such as 5-ketogluconic acid (12, 29) or condensation of two carbon fragments (8) remain to be explored experimentally.

Very little is also known of the mechanism of formation of tartaric acid in higher plants. Martin and Foster (15) found trans-L-epoxysuccinic acid in cultural filtrates of certain fungi maintained on glucose and were able to demonstrate the conversion of this acid to meso-tartaric acid by several fungi, bacteria, and a yeast. Although they proposed malic acid as a precursor of trans-L-epoxysuccinic acid, the present observations appear to rule out this possibility in higher plants. Further studies should, however, be made to learn if an epoxy intermediate formed in some other way is the immediate precursor of tartaric acid in plants.

Summary

$C^{14}O_2$ was fixed into malic, citric, tartaric, and some unidentified organic acids by excised grape leaves. Fixation into malic acid occurred both in the light and in the dark but into (+)-tartaric acid only in the light.

The highest specific activity of the tartaric acid was only about 1/1000 the value for malic acid and was obtained when three hours of light with $C^{14}O_2$ was followed by a 21-hour dark period in the absence of $C^{14}O_2$. After a continuous light treatment of 24 hours in the presence of $C^{14}O_2$, the tartaric acid had a lower specific activity. In the shortest light experiment, three hours, the internal carbons of tartaric acid were more highly labeled (75%) than the carboxyl carbons (25%), but when followed by a dark period or a continuous light treatment, the molecule became uniformly labeled. In contrast to this, in the short light experiment, the carboxyl carbons of malic acid were more highly labeled (85%) than the internal carbons (15%), becoming more uniformly labeled with longer treatment in the light or when a dark period followed the light. In the dark, only the carboxyl carbons of malic acid were labeled.

Enzyme preparations from grape leaves were similar to extracts from other leaves in that only (−)- and meso-tartrate were active in the diphosphopyridine nucleotide-dependent tartaric dehydrogenase system.

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Literature Cited

1. Allentoff, N., Phillips, W. R. and Johnston, F. B. A C^{14} study of carbon dioxide fixation in the apple. I. The distribution of incorporated C^{14} in the detached McIntosh apple. Jour. Sci. Food Agr. 5: 231–234. 1954.
2. Amerine, M. A. The maturation of wine grapes. Wines and Vines 37: (10)27–30, 32–36 and (11)53–55. 1956.
3. Amerine, M. A. and Winkler, A. J. Maturity studies with California grapes. II. The titratable acidity, pH, and organic acid content. Proc. Amer. Hort. Sci. 40: 313–324. 1942.
4. Bernstein, W. and Ballentine, R. Gas phase counting of low energy beta-emitters. Rev. Sci. Inst. 21: 158–162. 1950.
5. Blanchard, M. L., Korkes, S., Del Campillo, A. and Ochva, S. Function of biotin in the metabolism of *Lactobacillus arabinosus*. Jour. Biol. Chem. 187: 875–890. 1950.
6. Buch, M. L., Montgomery, R., and Porter, W. L. Identification of organic acids on paper chromatograms. Anal. Chem. 24: 489–491. 1952.
7. Coles, C. H. and Waygood, E. R. On the extraction of enzymes from succulent plants. Can. Jour. Bot. 35: 25–30. 1957.
8. Dickens, F. and Williamson, D. H. Formation of erythrulose from hydroxypyruvate in the presence of yeast carboxylase. Nature 178: 1349. 1956.
9. Genevois, L., Maurie, A., Peynard, F. and Ribereau-Gayon, J. Evolution des acides organiques chez la vigne. Congr. Intern. Bot. Paris; Rapp. et Commun. 8: 36–37. 1954.
10. Guichard, M. C. Contribution à l'étude des acides organiques chez les végétaux par chromatographie de portage sur papier. Rev. gén. bot. 62: 117–123. 1955.
11. Katz, J., Abraham, S. and Chaikoff, I. L. Analytical procedures using a combined combustion-diffusion vessel. An improved method for the degradation of C^{14}-labeled lactate and acetate. Anal. Chem. 27: 115–156. 1955.
12. Kheshgi, S., Roberts, H. R. and Bucek, W. Studies on the production of 5-ketogluconic acid by *Acetobacter suboxydans*. Appl. Microbiol. 2: 183–190. 1954.
13. La Riviere, J. W. M. Specificity of whole cells and cell-free extracts of *Pseudomonas putida* toward

(+), (−), and meso-tartrate. Biochim. Biophys. Acta 22: 206–207. 1956.
14. Loewus, F. A. and Stafford, H. A. Observations on the incorporation of C^{14} into tartaric acid and the labeling pattern of D-glucose from an excised grape leaf administered L-ascorbic acid-6-C^{14}. Plant Physiol. 33: 155–156. 1958.
15. Martin, W. R. and Foster, J. W. Production of trans-L-epoxysuccinic acid by fungi and its microbiological conversion to meso-tartaric acid. Jour. Bacteriol. 70: 405–414. 1955.
16. Nomura, M. and Sakaguchi, K. The decomposition of L(+)-tartrate by microorganisms. Jour. Gen. Appl. Microbiol. (Tokyo) 1: 77–98. 1956.
17. Palmer, J. K. Chemical investigations of the tobacco plant. X. Determination of organic acids by ion exchange chromatography. Connecticut Agr. Exp. Sta. Bull. 589: 1–31. 1955.
18. Perlin, A. S. Production of formic acid during oxidation of carbohydrates with lead tetraacetate. Jour. Amer. Chem. Soc. 76: 5505–5508. 1954.
19. Ranjan, S. and Rao, T. Roja. Separation of organic acids by circular paper chromatography: Variations in the organic acids content in Pelargonium. Naturwiss. 42: 581. 1955.
20. Shilo, M. The enzymic conversion of the tartaric acids to oxalacetic acid. Jour. Gen. Microbiol. 16: 472–481. 1957.
21. Shilo, M. and Stanier, R. Y. The utilization of the tartaric acids by Pseudomonads. Jour. Gen. Microbiol. 16: 482–490. 1957.
22. Sprinson, D. B. and Chargaff, E. On oxidative decarboxylations with periodic acid. Jour. Biol. Chem. 164: 433–449. 1946.
23. Stafford, H. A. Tartaric acid dehydrogenase activity in higher plants. Plant Physiol. 32: 338–345. 1957.
24. Stutz, R. F. and Burris, R. H. Photosynthesis and metabolism of organic acids in higher plants. Plant Physiol. 26: 226–243. 1951.
25. Van Slyke, D. D., Plazin, J. and Weisiger, J. R. Reagents for the Van Slyke-Folch wet carbon combustion. Jour. Biol. Chem. 191: 229–304. 1951.
26. Van Slyke, D. D., Steele, R. and Plazin, J. P. Determination of total carbon and its radioactivity. I. Jour. Biol. Chem. 192: 769–805. 1951.
27. Varner, J. E. and Burrell, R. C. Use of C^{14} in the study of the acid metabolism of *Bryophyllum calycinum*. Arch. Biochem. 25: 280–287. 1950.
28. Vaughn, R. H., Marsh, G. L., Stadtman, T. C. and Cantino, B. C. Decomposition of tartrates by the Coliform bacteria. Jour. Bacteriol. 52: 311–325. 1946.
29. Vickery, H. B. The metabolism of the organic acids of tobacco leaves. XIV. On the uptake of (+)-tartaric acid from solutions in the range pH 3 to pH 6. Jour. Biol. Chem. 227: 943–949. 1957.
30. Vickery, H. B. and Palmer, J. K. The metabolism of the organic acids of tobacco leaves. VII. Effects of culture of excised leaves in solutions of (+)-tartrate. Jour. Biol. Chem. 207: 275–285. 1954.

PROPERTIES OF ENOLASE IN EXTRACTS FROM PEA SEED[1,2]

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Enolase catalyzes the conversion of D-2-phosphoglyceric acid (2-PGA) to phosphoenolpyruvic acid (PEPA). The enzyme was first detected in muscle extract by Lohmann and Meyerhof (5), and later a crystalline mercury derivative was obtained by Warburg and Christian (14). The mercury compound was inactive as an enzyme but active enolase was obtained from the crystalline material by removal of the mercuric ion followed by the addition of Mg^{++}, Mn^{++}, or Zn^{++} (14). In the presence of phosphate, enolase from yeast (14), *Escherichia coli* (13) or animal tissues (15) is strongly inhibited by fluoride. It is believed that the normal activator of the enolase system is Mg^{++}, since the complex formed by magnesium, phosphate, and fluoride is only slightly dissociated.

Bonner and Wildman (1) inhibited respiration in spinach brei more than 90 % by the addition of 1 mg of NaF per ml. This inhibition was completely reversed by the addition of 2.5 mg of sodium pyruvate per ml, indicating the presence of the enolase system in spinach leaf brei. Stumpf (10) and Tewfik and Stumpf (11) provided further evidence for the presence of enolase in higher plants. The breakdown of PGA to acetaldehyde by pea extracts required Mg^{++} for activation and was sensitive to fluoride, indicating the involvement of the glycolytic enzymes known to catalyze these reactions.

The available literature on the enolase system in higher plants is limited, and properties of this system have not been elucidated. It was deemed of value to investigate and characterize the properties of this system in higher plants.

Materials and Methods

Preparation of Extracts: The enzyme extract used in these experiments was prepared from an acetone powder of *Pisum sativum* (Progress No. 9) seed. The seed was soaked in distilled water for five hours at room temperature; then removed from the water and homogenized for three minutes in acetone at − 10° C by use of an Omni mixer (Ivan Sorvall, Inc., Norwalk, Conn.). The acetone powder was prepared as previously described by Evans (2). For prepa-

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ration of the extract 1 g of acetone powder was homogenized in 40 ml of 0.05 M Tris (hydroxymethyl-aminomethane-hydrochloride) buffer at pH 7.4 and the mixture centrifuged at 25,000 × G for 10 minutes. The crude extract contained high enolase activity, but phosphoglyceric mutase and pyruvate kinase activity were also present. The extract was purified by $(NH_4)_2SO_4$ fractionation. Sufficient $(NH_4)_2SO_4$ was added to the supernatant to obtain a 30 % saturated solution. After centrifugation for 10 minutes at 25,000 × G, $(NH_4)_2SO_4$ was added to the supernatant until the solution was 55 % saturated. The $(NH_4)_2SO_4$ extract was centrifuged at 25,000 × G for 10 minutes and the precipitate taken up in 10 ml of TRIS buffer at pH 7.4. This extract was dialyzed for 24 hours against 4 liters of cold 0.01 M TRIS buffer at pH 7.4 and used as a source of enolase activity. High enolase activity was found in the purified extract but phosphoglyceric acid mutase, pyruvate kinase, 2-PGA phosphatase, and PEP-carboxykinase activity was not present in the standard reaction mixture. Storage of the extracts for one week at 0° C resulted in 60 % loss of enolase activity. The extracts prepared by the described procedure contained 5 to 6 mg protein per ml as determined by Folin's phenol reagent (6). The $(NH_4)_2SO_4$ fractionated extract was a 7-fold purification over the crude extract.

DL-2-PHOSPHOGLYCERIC ACID: Phosphoglyceric acid was synthesized from β-glycerophosphate (Eastman Chemicals, Rochester, N. Y.) according to the method of Kiessling (4). The product was purified according to procedure outlined by Warburg and Christian (14). After purification less than 10^{-4} M free phosphate was present. Heavy metals were removed with a solution of dithizone in CCl_4 as described by Malmström (7). The concentration of 2-PGA was determined by hydrolysis of the phosphate group (12), and total phosphorus was determined by the method of Fiske and SubbaRow (3).

STANDARD ASSAY PROCEDURE: Enzyme activity was measured by following the change in optical density of the test solution at 240 mμ as described by Warburg and Christian (14). Five hundredth ml of enzyme solution was added to 1 ml of substrate solution and the O.D. determined every 30 seconds over the desired time range. Since the rate of reaction remained linear for at least 10 minutes under the specified conditions, enzyme activity was calculated as O.D. change in the initial minute at 240 mμ.

The reaction mixture contained the following constituents, expressed as micromoles per ml: 50 TRIS buffer at pH 8.0, 5 2-PGA, 1.0 $MgSO_4$, 30 glycine, and the enzyme extract usually containing 0.1 mg protein. Glycine was included to bind traces of copper (14). The various experiments were replicated three times and the results averaged. Error between determinations was less than 10 %.

A Beckman DU spectrophotometer was used with 1-cm silica cells. The housing of the absorption cells was far enough from the hydrogen lamp to prevent heating. Since the absorption of PEPA changes rapidly with wave length (7) the spectral purity must be high. The spectral band width obtained with the Beckman DU was about 17 Å at 240 mμ. Serial dilutions of PEPA gave a straight line when the optical density was plotted as a function of the concentration, showing that the band width of the Beckman DU was sufficiently small.

RESULTS

NATURE OF THE SYSTEM: As shown in figure 1, a concentration of 5×10^{-3} M 2-PGA was necessary to saturate the enzyme. The Michaelis constant (K_M) for 2-PGA estimated from the saturation curve is 2.5×10^{-4} M. Conversion of 3-PGA to PEPA occurred with an undialyzed extract, but the rate of reaction was less than 10 % of that observed utilizing 2-PGA as the substrate. In the presence of a dialyzed extract 3-PGA was completely ineffective as a substrate for the reaction.

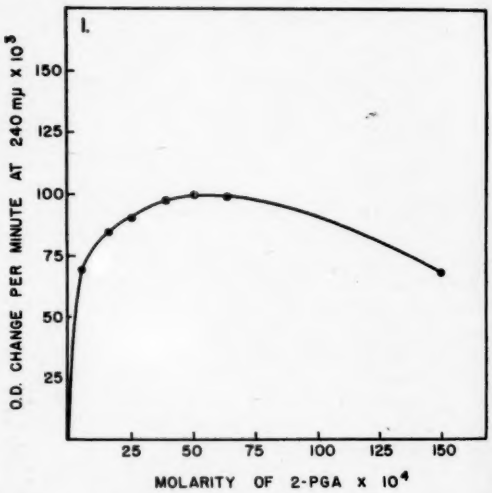
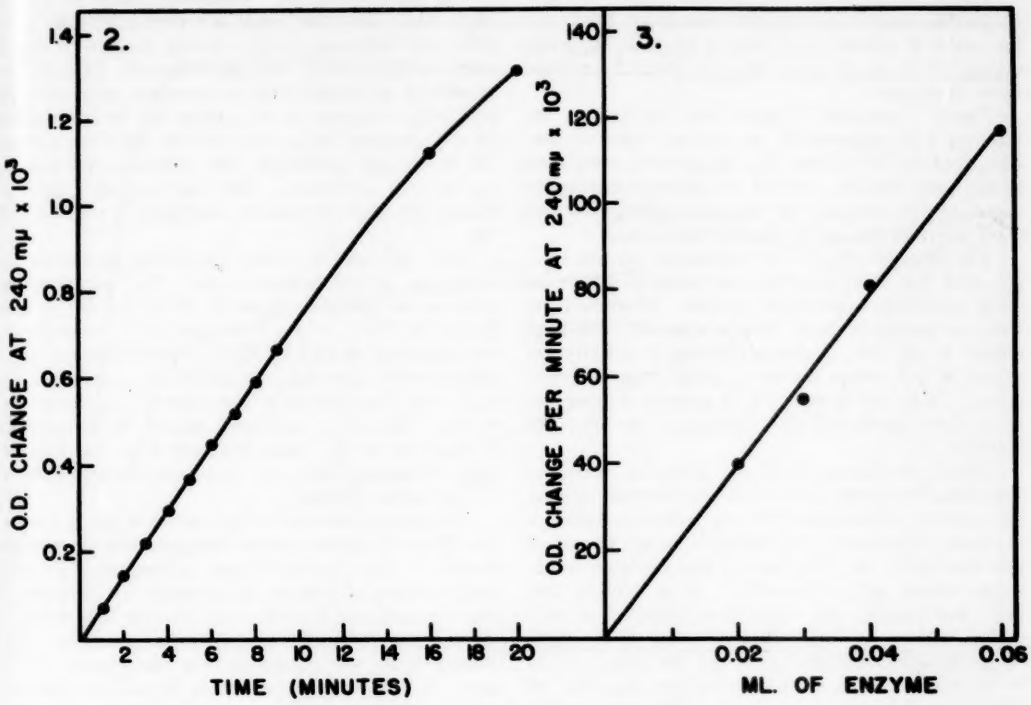


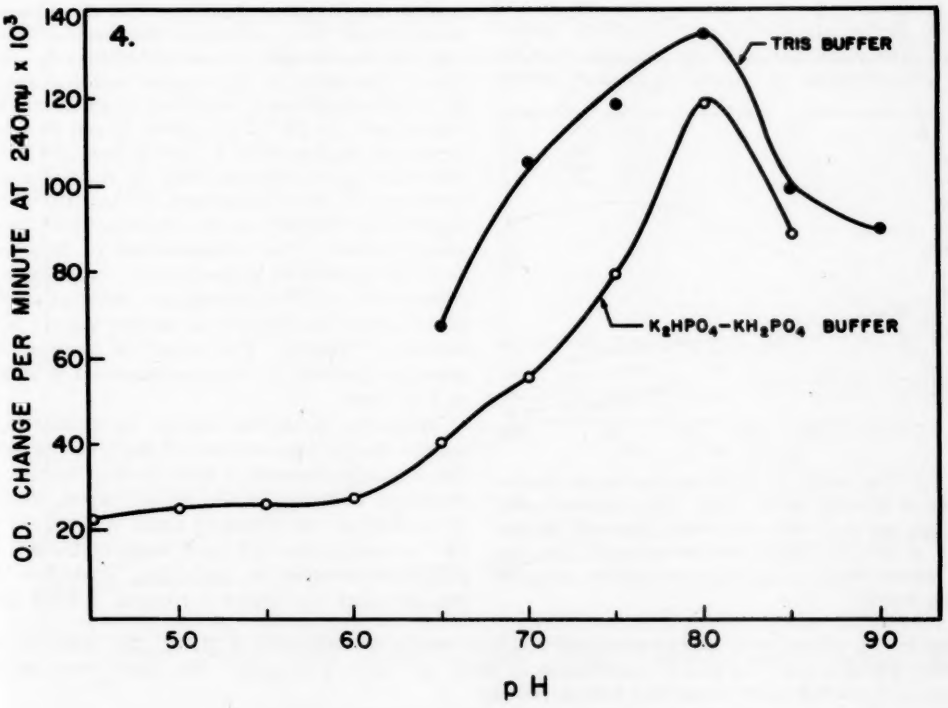
FIG. 1. The rate of enzyme reaction as related to concentration of 2-PGA. The standard assay procedure was used with variation in concentration of 2-PGA as indicated. The extract from pea seed added to each reaction mixture contained 0.13 mg protein.

FIG. 2. Proportionality of enzyme activity with time. The standard assay procedure was used with variation in time as indicated. The enzyme extract added to each reaction mixture contained 0.09 mg protein.

FIG. 3. Proportionality of enzyme activity with enzyme concentration. The standard assay procedure was used with variation in quantity of extract added as indicated. The extract contained 1.0 mg protein per ml.

FIG. 4. Enolase activity as related to pH. The standard assay procedure was used with variation in the pH value as indicated. The enzyme extract added to the reaction mixture contained 0.14 mg protein.


2.
O.D. CHANGE AT 240 mμ x 10³
1.4
1.2
1.0
0.8
0.6
0.4
0.2
2 4 6 8 10 12 14 16 18 20
TIME (MINUTES)
3.
O.D. CHANGE PER MINUTE AT 240 mμ x 10³
140
120
100
80
60
40
20
0.02 0.04 0.06
ML. OF ENZYME


4.
O.D. CHANGE PER MINUTE AT 240mμ x 10³
140
120
100
80
60
40
20
TRIS BUFFER
K₂HPO₄–KH₂PO₄ BUFFER
5.0 6.0 7.0 8.0 9.0
pH

Under conditions of the standard assay the rate of PEPA formation remained essentially linear for the initial 10 minutes as shown in figure 2. Approximately 10 % of the total substrate was hydrolyzed after 10 minutes.

Figure 3 provides evidence that the rate of the reaction was proportional to enzyme concentration. Low amounts of enzyme (0.1 mg protein) were used in the assay medium. PEPA accumulated during the course of the reaction and was not broken down by other enzymes present in the dialyzed extract.

The effect of pH on the activity of enolase from pea seed was studied within the range of 4.5 to 9.0 using phosphate and TRIS buffers. Maximum activity as shown in figure 4 was observed with both buffers at pH 8.0. A sharp decrease in activity occurred at pH values higher or lower than the optimum. TRIS buffer resulted in greater enzyme activity than phosphate buffer throughout the pH range studied.

Effect of Metal Ions on Enzyme Activity: The effect of various divalent cation chlorides on enolase activity is illustrated by the curves in figure 5. In these experiments the standard assay procedure was used with the exception of the variation in divalent cation salt as described. It is evident that Mn^{++} and possibly Co^{++} were more effective in activating enolase at lower concentrations than Mg^{++}. Warburg and Christian (14) found Mn^{++} and Zn^{++} to be more effective at low concentrations than Mg^{++} in activation of enolase from yeast. Maximum activity was obtained with Mg^{++}, Mn^{++}, Co^{++}, and Zn^{++} at 10^{-3} M, 10^{-4} M, 4×10^{-4} M and 3×10^{-5} M, respectively. The maximum activities measured with the same concentrations of enzyme (maximum activity in the presence of Mg^{++} arbitrarily taken as 1) for Mg^{++}, Mn^{++}, Co^{++}, Zn^{++} and Ca^{++}, were 1.00, 0.41, 0.30, 0.07, and 0.00 respectively. In the absence of added salts enzyme activity was approximately 15 % of the maximum attainable with an optimum concentration of $MgCl_2$. Dialysis of the extract for longer periods of time resulted in enzyme activity less than 5 % of the maximum attainable, but complete dependence on salt was not shown. The half saturation concentration for Mg^{++} calculated from figure 5 was 2×10^{-4} M.

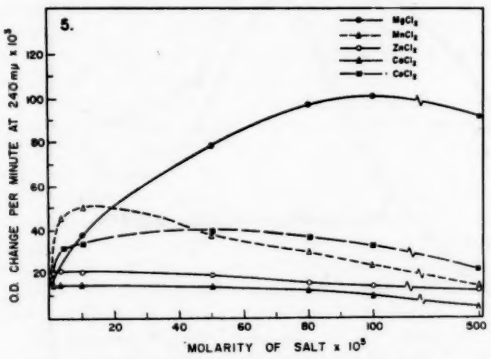


Fig. 5. The activation of enolase by various concentrations of divalent cation salts. The standard assay procedure was used with variation in type and concentration of divalent cation salts as indicated. The enzyme extract added to each reaction mixture contained 0.10 mg protein.

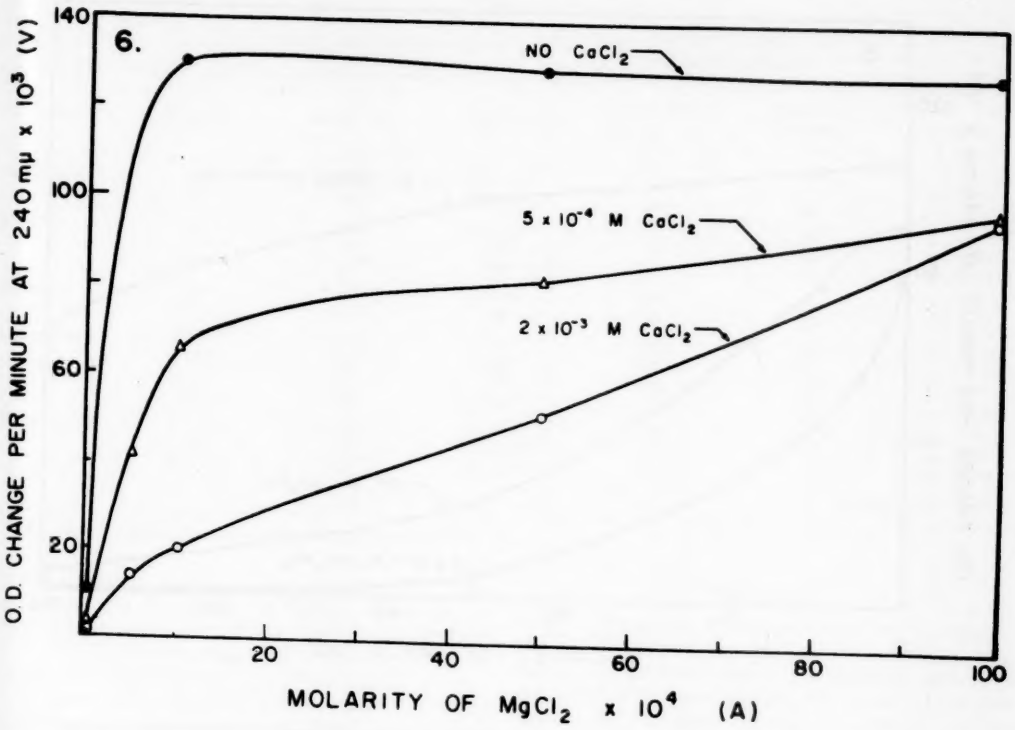
Ca^{++}, K^{+}, and Na^{+} were completely ineffective as activators for the enolase system. Ca^{++} inhibited the reaction as shown in figure 6. With 2×10^{-3} M and 5×10^{-4} M Ca^{++}, 85 and 50 % inhibition, respectively, was exhibited at 10^{-3} M $MgCl_2$. Increasing the Mg^{++} concentration decreased the inhibition manifested by Ca^{++} until the inhibition was almost completely reversed. The curves in figure 7 plotted by the method of Lineweaver and Burk obtained from the data of figure 6 indicate that Ca^{++} is a competitive inhibitor of the enolase system.

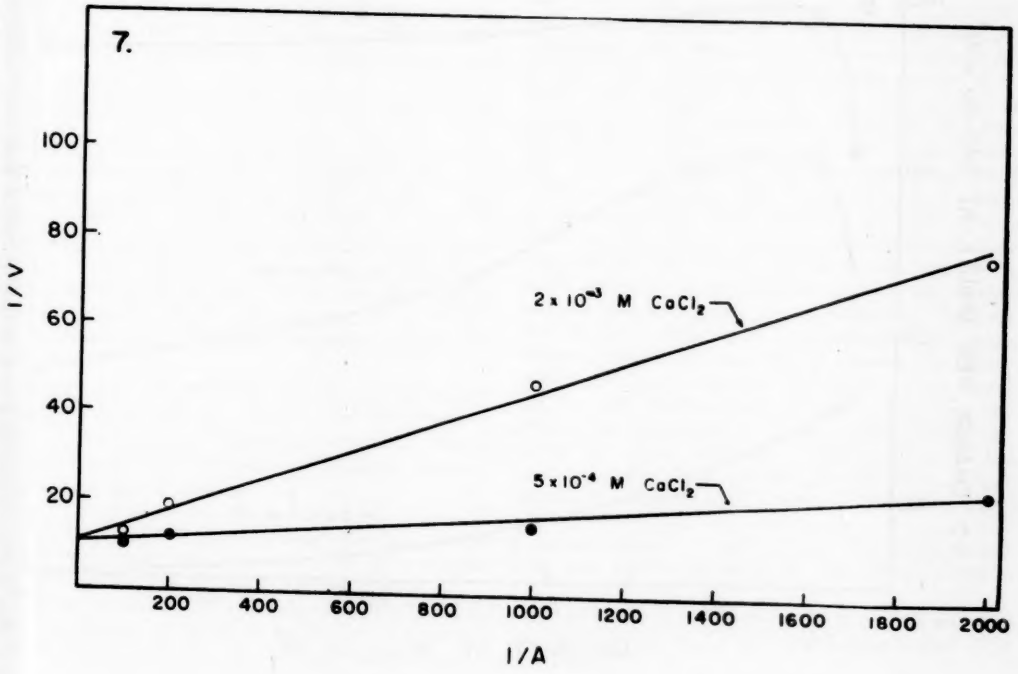
Fluoride Inhibition: The curves in figure 8 show the effect of various fluoride concentrations on enolase activity. The standard assay procedure was used with addition of fluoride as indicated. It was necessary to incubate fluoride with the reaction mixture for one minute before full inhibition developed. Enzyme activity was calculated from the second minute since the activity remained linear for several minutes. The reaction mixture contained less than 10^{-4} M endogenous inorganic phosphate. At this phosphate concentration 10 % inhibition was manifested at 5×10^{-3} M fluoride and 41 % at 10^{-2} M fluoride. Addition of phosphate to the reaction mixtures resulted in a marked increase in inhibition at all fluoride concentrations. At 10^{-3} M phosphate 79 and 95 % inhibition was manifested at 5×10^{-3} M and 10^{-2} M fluoride, respectively; whereas when the reaction mixture contained 5×10^{-3} M phosphate, 95 and 100 % inhibition were obtained at the aforementioned fluoride concentrations. Low concentrations of fluoride (5 to 10 ppm) inhibited enolase activity markedly in the presence of 5×10^{-3} M phosphate. Addition of phosphate resulted in inhibition of enolase activity in the absence of fluoride. This would be expected since phosphate reduces the effective concentration of Mg^{++} as a cofactor.

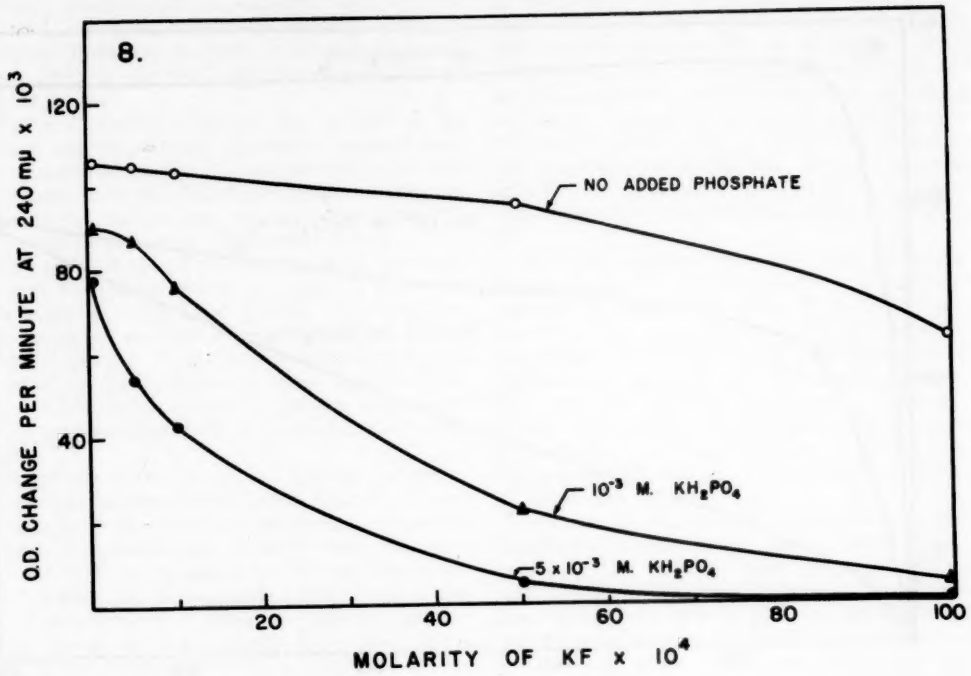
Inhibition of enzyme activity by fluoride is dependent on the concentration of Mg^{++}, but increasing the Mg^{++} concentration at fixed levels of fluoride and phosphate does not, as one would suspect, decrease the inhibition. As shown in figure 9 increasing the Mg^{++} concentration with fixed levels of fluoride and phosphate increases the inhibition. At higher Mg^{++} concentrations less fluoride is required to bring about

Fig. 6. The inhibition of enzyme activity by $CaCl_2$ at various concentrations of $MgCl_2$. The standard assay procedure was used with variations in concentration of $MgCl_2$ and $CaCl_2$ as indicated. The extract from pea seed added to each reaction mixture contained 0.12 mg protein.

Fig. 7. The data of figure 6 plotted by the method of Lineweaver and Burk.


6.
NO $CaCl_2$
5×10^{-4} M $CaCl_2$
2×10^{-3} M $CaCl_2$
O.D. CHANGE PER MINUTE AT 240 mμ x 10^3 (V)
MOLARITY OF $MgCl_2$ x 10^4 (A)
140
100
60
20
20
40
60
80
100


7.
2×10^{-3} M $CaCl_2$
5×10^{-4} M $CaCl_2$
1/V
1/A
100
80
60
40
20
200
400
600
800
1000
1200
1400
1600
1800
2000


8.
O.D. CHANGE PER MINUTE AT 240 mμ x 10³
120
80
40
NO ADDED PHOSPHATE
10⁻³ M. KH₂PO₄
5 x 10⁻³ M. KH₂PO₄
20
40
60
80
100
MOLARITY OF KF x 10⁴

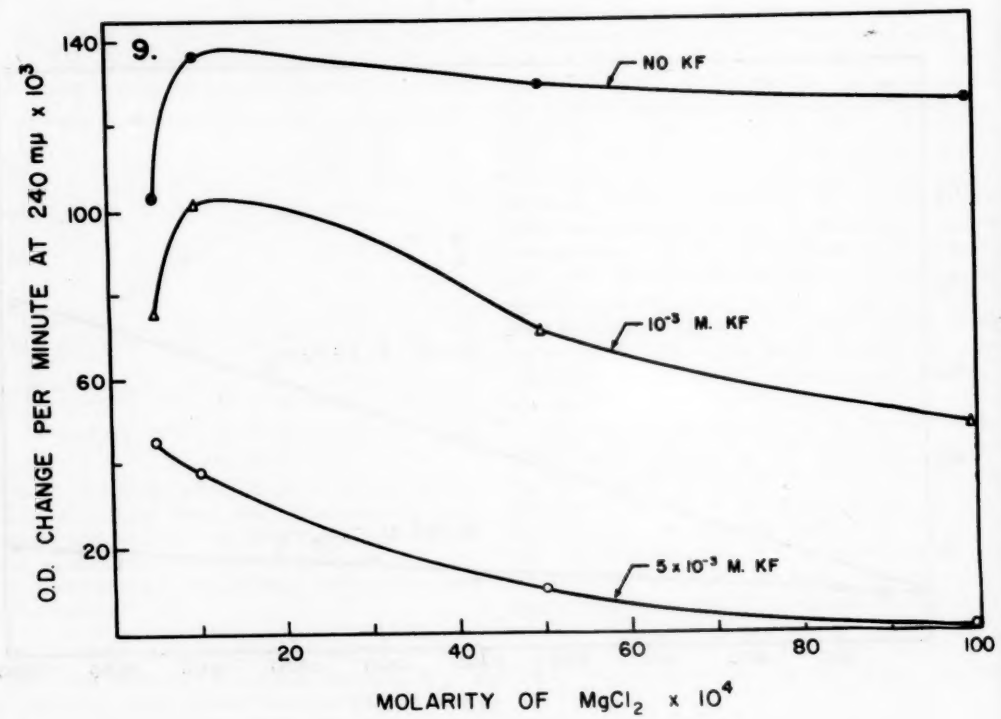

9.
O.D. CHANGE PER MINUTE AT 240 mμ x 10³
140
100
60
20
NO KF
10⁻³ M. KF
5 x 10⁻³ M. KF
20
40
60
80
100
MOLARITY OF MgCl₂ x 10⁴

TABLE I

CALCULATION OF CONSTANT (k) = $C_{Mg} \cdot C_{PO_4} \cdot C^2_F \cdot \frac{\text{RESIDUAL ACTIVITY}}{\text{INHIBITED ACTIVITY}}$

VARIATION	C_{Mg} (M)	C_{PO_4} (M)	C_F (M)	INHIBITED ACTIVITY %	RESIDUAL ACTIVITY %	k (M^4)
Phosphate	10^{-3}	5×10^{-3}	5×10^{-3}	94	6	8×10^{-12}
	10^{-3}	10^{-3}	5×10^{-3}	70	30	11×10^{-12}
Fluoride	10^{-3}	10^{-3}	5×10^{-4}	5	95	5×10^{-12}
	10^{-3}	10^{-3}	10^{-3}	17	83	5×10^{-12}
	10^{-3}	10^{-3}	10^{-2}	94	6	6×10^{-12}
Fluoride	10^{-3}	5×10^{-3}	5×10^{-4}	31	69	3×10^{-12}
	10^{-3}	10^{-3}	10^{-3}	45	55	6×10^{-12}
Magnesium	5×10^{-4}	10^{-3}	10^{-3}	25	75	2×10^{-12}
	10^{-3}	10^{-3}	10^{-3}	26	74	3×10^{-12}
	5×10^{-3}	10^{-3}	10^{-3}	45	55	6×10^{-12}
	10^{-2}	10^{-3}	10^{-3}	54	46	8×10^{-12}
Magnesium	5×10^{-4}	10^{-3}	5×10^{-3}	56	44	10×10^{-12}
	10^{-3}	10^{-3}	5×10^{-3}	72	28	10×10^{-12}
	5×10^{-3}	10^{-3}	5×10^{-3}	92	8	11×10^{-12}
						Average = 7×10^{-12}

* Data of figures 8 and 9 used to calculate constant (k).

a certain level of inhibition than at lower Mg^{++} concentrations. In the absence of fluoride maximum activity is observed at 10^{-3} M Mg^{++} but in the presence of 5×10^{-3} M fluoride, maximum activity is found at a lower Mg^{++} concentration.

Warburg and Christian (14) observed with crystalline enolase from yeast that fluoride inhibition resulted from the formation of a magnesium-fluorophosphate complex. In the range of fluoride concentrations from 10^{-2} to 10^{-4} M the inhibition was correlated with the following relationship: $C_{Mg} \cdot C_{PO_4} \cdot C^2_F \cdot \frac{\text{Residual activity}}{\text{Inhibited activity}} = 3.2 \times 10^{-12}\ M^4$, where C is the concentration of the indicated ion. Calculations of the data from figures 8 and 9 using this relationship are shown in table I. The calculated constant varied from 2×10^{-12} to 11×10^{-12} and averaged 7×10^{-12}. Through the use of this constant and the data represented by the upper curve in figure 8, the phosphate concentration in the medium with no added phosphate was calculated. The value calculated, 4×10^{-5} M, agrees closely with the value determined experimentally (3).

A survey of several plant species showed enolase activity to be present in extracts from the leaves of *Pisum sativum*, seed of *Gossypium barbadense*, leaves of *Chenopodium murale*, leaves of *Nicotiana tabacum* and seeds of *Avena sativa*.

DISCUSSION

The properties of enolase present in an acetone powder of pea seed are similar to those of the crystalline enzyme from yeast. The observed differences are perhaps in part due to the degree of enzyme purification. Divalent cations were activators of the enolase system from higher plants. Magnesium, Mn^{++}, Co^{++}, and, to a slight extent, Zn^{++} activated this system in the ratio of 1.00 : 0.41 : 0.30 : 0.07, respectively. This compares to the activating capacity of Mg^{++}, Mn^{++} and Zn^{++} on crystalline enolase of 1 : 0.425 : 0.419, respectively (7). The effect of Co^{++} on enolase from other sources has apparently not been determined. Maximum activity was obtained with Mg^{++} at 10^{-3} M and with the other divalent cations at lower concentrations. The residual activity observed in the absence of added salts was probably due to traces of Mg^{++} present in the reaction mixture. Less than 10^{-5} M Mg^{++} would have accounted for the residual activity. In the presence of Ca^{++} the residual activity became essentially zero, indicating the presence of Mg^{++}. Addition of a concentration of divalent cations greater than the amount needed for saturation resulted in a decrease in activity. That this is not an ionic phenomenon is indicated by the differences in concentration of the various cations required for above saturation of the enzyme. It has been suggested (7, 8, 9) that the inhibition at high activator concentrations is due to the electro-static

FIG. 8. The inhibition of enolase activity by fluoride at various phosphate concentrations. The standard assay procedure was used with variations in the concentration of KF and KH_2PO_4 as indicated. The extract added to each reaction mixture contained 0.08 mg protein.

FIG. 9. Enolase activity as related to $MgCl_2$ and KF concentrations. The standard assay procedure was used with the addition of 10^{-3} M phosphate and variations in the concentration of KF and $MgCl_2$ as indicated. The extract added to each reaction mixture contained 0.11 mg protein.

effect of activating ions bound to nonspecific sites. Malmström suggested that the function of magnesium in the enzymatic catalysis is mainly twofold: (a) to furnish one of at least two points of interaction between enzyme and substrate; and (b) to modify the structure of the active site on the enzyme surface. The K_A value calculated for Mg^{++} was 2×10^{-4} M. Warburg and Christian (14) observed saturation of enolase with Mg^{++} at 2.8×10^{-3} M. Malmström showed K_A values increased with an increase in 2-PGA, phosphate concentration, or pH (9). He observed a K_A value of 2.9×10^{-4} M for Mg^{++} in TRIS buffer at pH 7.9.

Fluoride inhibition of the enolase system from pea was found to be dependent on the magnesium, phosphate, and fluoride concentrations. Marked inhibition was manifested at 5 to 10 ppm fluoride in the presence of relatively high concentrations of magnesium and phosphate. The constant calculated according to Warburg and Christian (14), $k = C_{Mg} \cdot C_{PO_4} \cdot C^2_F \cdot \frac{\text{Residual activity}}{\text{Inhibited activity}}$ was found to be about 3×10^{-12} M^4. The constant (k) from the data in our experiments was 7×10^{-12} M^4. Greater purification of the enzyme from pea seed would be necessary to determine the absolute value of k. Mg^{++} serves as an activator for the enolase system and the ion attached to some specific site on the enzyme protein activates the system (14). It seems likely that the magnesium-fluorophosphate complex occupies this same site thus inactivating the enzyme. The inhibition can be reversed by removal of the complex groups through dialysis and addition of Mg^{++} to the system.

There have been many reports of injury to plants that have been exposed to fluoride in the atmosphere or in the soil (15). This injury is manifested by susceptible plant species such as *Chenopodium murale* at concentrations of atmospheric fluoride as low as 0.4 parts per billion. Plant injury may be related to the observed fluoride inhibition of the enolase system. It would be logical to expect that blocking the enolase reaction in plants not containing an alternate pathway would affect metabolism in general. Plants are now being grown in fluoride fumigation chambers and a comparison of the enolase activity between normal and fumigated plants is being made. The results of these observations will be reported later.

Summary

Experiments were conducted to determine the properties of enolase from higher plants. The concentration of 2-PGA required for saturation of the enzyme from a dialyzed extract of pea seed was determined. After the cation requirements were established evidence was obtained to show that enzyme activity was proportional to both enzyme concentration and reaction time under the specified conditions.

Detailed studies of the system demonstrated a cation requirement that was satisfied by Mg^{++} at 10^{-3} M. Mn^{++}, Co^{++} and Zn^{++} activated the enzyme reaction, but were much less effective than Mg^{++}. Ca^{++} was found to be a competitive inhibitor, reversal of the inhibition being achieved by increasing the Mg^{++} concentration.

Fluoride inhibition of the enolase system in pea seed extracts was dependent on the fluoride, phosphate and magnesium concentration. Using the relationship of Warburg and Christian (14) concerning fluoride inhibition, a constant (k) was calculated and was similar to that observed for crystalline enolase.

Personal communication with Dr. M. D. Thomas, Stanford Research Institute revealed that he and Dr. S. L. Chen, Red Star Yeast Co., had completed experiments on the properties of purified enolase from barley in 1954. This work is now in the process of being submitted for publication.

Literature Cited

1. Bonner, J. and Wildman, S. G. Enzymatic mechanisms in the respiration of spinach leaves. Arch. Biochem. 10: 497–517. 1946.
2. Evans, H. J. Studies on cytochrome reductase in higher plants. Plant Physiol. 30: 437–444. 1955.
3. Fiske, C. H. and SubbaRow, Y. Colorimetric determination of phosphorous. Jour. Biol. Chem. 66: 375–400. 1925.
4. Kiessling, W. Die Synthese der isomeren Glycerinsäure-phosphorsäuren (Phosphoglycerinsäure). Ber. deut. chem. Ges. 68: 243–249. 1935.
5. Lohman, K. and Meyerhof, O. Enzymatic transformation of phosphoglyceric acid into pyruvic and phosphoric acids (translated title). Biochem. Zeits. 273: 60–73. 1934.
6. Lowry, D. H., Rosebrough, Nira J., Farr, A. L. and Randall, Rose J. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275. 1951.
7. Malmström, B. G. The interaction of purified enolase with its activating metal ions. Arch. Biochem Biophys. 46: 345–363. 1953.
8. Malmström, B. G. The interaction of DL-2-phosphoglyceric acid with metal ions activating enolase. Arch. Biochem. Biophys. 49: 335–343. 1954.
9. Malmström, B. G. and Westlund, L. E. The effect of pH on the interaction of enolase with activating metal ions. Arch. Biochem. Biophys. 61: 186–195. 1956.
10. Stumpf, P. K. Carbohydrate metabolism in higher plants. III. Breakdown of fructose diphosphate by pea extracts. Jour. Biol. Chem. 182: 261–272. 1950.
11. Tewfik, S. and Stumpf, P. K. Carbohydrate metabolism in higher plants. IV. Observations on triose phosphate dehydrogenase. Jour. Biol. Chem. 192: 519–526. 1951.
12. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. Manometric Techniques and Tissue Metabolism. Burgess, Minneapolis 1951.
13. Utter, M. F. and Werkman, C. H. Dissimilation of phosphoglyceric acid by *Escherichia coli*. Biochem. Jour. 36: 485–493. 1942.
14. Warburg, O. and Christian, W. Isolierung and Kristallisation des Gärungsferment Enolase. Biochem. Zeits. 310: 385–421. 1942.
15. Zimmerman, P. W. Proc. U. S. Tech. Conf. on Air Pollution. Pp. 127–139. McGraw-Hill, New York 1952.

ENZYMATIC FORMATION OF INDOLE-3-CARBOXALDEHYDE FROM INDOLE-3-ACETIC ACID[1,2]

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It was first suggested by Tang and Bonner (14) that indole-3-carboxaldehyde is the product of the oxidative degradation of indole-3-acetic acid (IAA) by crude enzyme preparations from etiolated pea seedlings. Using the more active bean root enzyme, Wagenknecht and Burris (15) attempted without success to obtain the 2,4-dinitrophenylhydrazone of the aldehyde. On the basis of chromatographic data, Racusen (9) reports that the pea enzyme forms small amounts of the aldehyde. More recently, Wagenknecht (personal communication) has made similar observations with the bean root system. Ray and Thimann (10) and Ray (11) have recently reported that the enzyme from *Omphalia flavida* is unable to form the aldehyde. The recent work of Maclachlan and Waygood (6) likewise has left unsettled the nature of the end product.

Manning and Galston (7) have reported the formation in the pea system of two products having high R_f values and giving qualitative color tests which indicate a similarity to our products # 4 and # 5 (see below). They were unable to demonstrate the formation of indole-3-carboxaldehyde, and also ruled out *ortho*-formamidoacetophenone, *ortho*-aminoacetophenone, and 4-hydroxyquinoline as major end products, although they believe that the indole ring is ruptured.

Chromatographic separation and spectrophotometric examination of the products of the IAA oxidase-peroxidase system from *Lupinus albus* L. (13) gave no indication that the aldehyde was formed; likewise, the results obtained when the chromatograms were tested with 2,4-dinitrophenylhydrazine hydrochloride, modified Salkowski (3), and Ehrlich reagent suggested the absence of the aldehyde. In view of the conflicting results, attempts were made to supplement the purified IAA oxidase with the auxiliary enzyme systems presumed to be present in the crude enzyme preparations employed in the early investigations. The present report is concerned with the coupling of the oxidase system to the cytochrome oxidase system. Under these conditions the formation of indole-3-carboxaldehyde as a major reaction product was observed.

Subsequent reexamination of the reaction products from the oxidase-peroxidase system, particularly those freshly prepared from etiolated lupine hypocotyls, indicated the normal formation of the aldehyde in small amounts. Therefore, in order to obtain data on the oxidation mechanism, indole-3-carboxylic acid and a number of substituted indole-3-acetic acid derivatives were tested both with the oxidase-peroxidase system alone and with the added cytochrome system. It is of interest to note that since this paper was submitted, Jones and Taylor (5) have reported the isolation of both indole-3-carboxaldehyde and indole-3-carboxylic acid from cabbage extracts.

MATERIALS AND METHODS

Preparation and purification of the IAA oxidase from *Lupinus albus* L. has been previously described (13). The course of the oxidation of IAA was followed with the Warburg manometric apparatus under the conditions outlined earlier (13) except where 0.4 M tris-(hydroxymethyl)-aminomethane buffer at pH 7.2 was used in an attempt to approach the pH optimum of the cytochrome oxidase system and still maintain IAA oxidase activity. Under these conditions a small amount of phosphate, 4 micromoles per flask, was added for optimum IAA oxidase activity. The normal 3-ml reaction mixture was enzyme; 1 ml 0.4 M orthophosphate buffer, pH 6.3; 0.3 micromole $MnCl_2$; 0.3 micromole sodium-2,4-dichlorophenolate; (in the main compartment) 0.1 ml M KOH with a standard wick in the center well; and 30 micromoles of sodium indole-3-acetate in the side arm. When the cytochrome system was to be coupled to the oxidase, cytochrome oxidase was added to each flask in the form of 0.2 ml of a 5% suspension of homogenized rat liver, together with cytochrome c, usually 2×10^{-5} M, and 0.3 micromoles of Al^{+++}.

Ascending chromatography on Whatman 3-mm filter paper was carried out with a solvent mixture consisting of 28% NH_4OH, water, and isopropyl alcohol in proportions ranging from 1 : 1 : 6 to 1 : 1 : 12, depending on whether it was desired to obtain greater resolution of the more acidic or the neutral fraction of the products. The reaction products to be discussed have been designated by numbers 1 to 5 in order of increasing R_f value (13). This has been done in preference to R_f designations alone because the relative positions of the components were retained in the various solvent mixtures used. The products (13) which appeared important in this study were the apparently large # 2 (R_f 0.6) group of non-indolyl fluorescent compounds and the # 4 and # 5 (R_f 0.8 and 0.9), presumably polymeric indolyl, products which bracket the aldehyde. The # 1 (R_f 0.45) indolyl component (13) was not encountered in this study—presumably because the reaction with crude enzyme was not permitted to go to exhaustion.

In some experiments, the substrate was IAA, labeled with C^{14} either in the methylene (12) or in the carboxyl position (13). In order to obtain suffi-

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cient activity on the chromatograms to permit reasonable counting time and accuracy, it was found necessary to label the substrate in each flask with approximately 30 microcuries of the methylene-labeled IAA or 3 microcuries of carboxyl-labeled IAA. C^{14} in the reaction products was measured by counting the chromatograms directly. Sixth-tenths of a milliliter of the enzyme reaction mixture was applied to a 4-inch segment of the chromatograph sheet. After chromatographic separation, sections 6 cm wide were counted by advancing the strip 1 cm at a time through the masked chamber of a modified PCC-10 proportional counter (Nuclear Measurements Corp., Indianapolis, Ind.).

Qualitative survey of the products on the chromatograms from all of the Warburg experiments was made by ultraviolet examination (Mineralight SL 2537) and by swabbing the strips with Ehrlich's reagent, 2,4-dinitrophenylhydrazine hydrochloride, and modified Salkowski reagent (3). The sensitivity of the ultraviolet scanning was greatly enhanced by illuminating the chromatograms from the back. Quantities of the indole-3-carboxaldehyde which were normally undiscernible were thus rendered detectable.

Fractionation and colorimetric determination of indole-3-carboxaldehyde and other indole products in the reaction mixture was carried out by the following procedure. One ml of the reaction mixture from the Warburg flask was added to 9 ml of buffer containing 7.5 % $NaHCO_3$ and 2.5 % Na_2CO_3, and extracted with three 15-ml portions of peroxide-free ether. IAA was estimated in the aqueous phase with the modified Salkowski reagent (3) after the bulk of the dissolved ether had evaporated. Although the inadequacies of the Salkowski method have been well documented by previous investigators (8), the procedure was used in an attempt to obtain an evaluation of the residual IAA and the IAA equivalents of some of the products. It had been hoped that the Salkowski data along with the aldehyde and oxygen uptake values would permit an approximation of the reaction balance.

For the determination of indole-3-carboxaldehyde, the ether phase was evaporated to dryness in an air stream and the residue was taken up in 5 ml of absolute ethanol. A suitable aliquot of the ethanol solution was made up to 5 ml with ethanol, and to this was added 0.5 ml of 2,4-dinitrophenylhydrazine hydrochloride reagent (100 mg of 2,4-dinitrophenylhydrazine hydrochloride in 100 ml of 6 *N* HCl). The colored solution was read in the Klett photoelectric colorimeter, using the No. 54 filter. Color development is instantaneous; the color is stable for at least an hour and apparently is unaffected by the presence of other indolyl compounds. The standard curve for indole-3-carboxaldehyde has a range of 0 to 200 micrograms over 340 Klett units. Recovery values in samples added to the Warburg reaction mixture substantiated the reliability of the extraction method.

Purification of the indole-3-carboxaldehyde for identification was carried out by strip chromatography of the fraction extractable from the reaction mixture by ether at pH 10. The aldehyde fraction was eluted with acetone, concentrated, and the chromatography repeated. A small amount was taken up in ethanol for the ultraviolet absorption measurement; another aliquot was mixed with powdered KBr and a disk was prepared for infrared spectrophotometry. The invaluable help of Dr. J. H. Pomeroy and Mrs. C. A. Craig in this phase of the work is gratefully acknowledged.

Since indole-3-carboxaldehyde does not react appreciably with modified Salkowski reagent, the latter was used to determine the non-aldehyde, Salkowski-positive, indolyl compounds soluble in ether at pH 10 (the # 4 and # 5 products on the chromatograms), and a survey was made by comparison with the IAA standard curve.

The author wishes to acknowledge the provision of the substituted IAAs so generously made available for this investigation. Na-indole-3-glycolate was supplied by Dr. Marvin D. Armstrong of the University of Utah. Ethylindole-3-glyoxylate was obtained from Dr. Bruce Stowe of Harvard University; the ester was hydrolyzed by the method of Elks et al (1). Ethylindole-3-isobutyrate was provided by Dr. H. Erdtman and Dr. Å. Jönsson of the Tekniska Högskolan, Stockholm, Sweden; the free acid was prepared by their method (2). Samples of 1-methyl-IAA, 2-methyl-IAA, and 2-carboxy-IAA were obtained from Dr. Sidney Fox of the University of Florida. Indole-3-carboxylic acid was supplied by Dr. Norman E. Good of the Science Service Laboratory, London, Ontario, Canada. Dr. K. Mann of the Upjohn Laboratories, Kalamazoo, Michigan, provided

TABLE I

RESPONSE OF IAA OXIDASE TO THE CYTOCHROME SYSTEM

IAA-oxidase preparation	Cytochrome c	Oxygen uptake *	IAA used	Product # 4 **	Indole-3-carboxaldehyde
ml	*moles*	*micro-moles*	*micro-moles*	*micro-moles*	*micro-moles*
0.0	1×10^{-4}	−0.49	0.9	0.02	0.21
0.5	1×10^{-4}	0.22	0.9	0.1	0.62
"	5×10^{-5}	2.9	6.35	0.34	2.69
"	1×10^{-5}	9.8	14.7	0.79	4.0
"	5×10^{-6}	11.2	15.8	1.03	3.52
"	1×10^{-6}	14.3	18.9	1.17	2.28
"	0×0	13.9	16.6	1.05	0.83
1.0	1×10^{-4}	−0.31	1.43	0.07	0.62
"	5×10^{-5}	2.2	6.0	0.34	2.28
"	1×10^{-5}	14.5	20.0	1.35	4.35
"	5×10^{-6}	17.5	21.7	1.7	4.14
"	1×10^{-6}	18.0	22.1	1.66	3.2
"	0×0	17.3	21.1	1.58	1.79

* These values were obtained after 90 min at 30° C. The amounts of reaction products listed were obtained from 3.0 ml reaction mixture. Flasks containing cytochrome c also contained 0.2 ml of 5 % rat liver homogenate.

** The estimation of reaction product #4 is described under Materials and Methods.

the 5-hydroxy-IAA. The indole-3-carboxaldehyde was obtained from Dow Chemical Company, Midland, Michigan.

Results and Discussion

Indole-3-carboxaldehyde has for a long time figured prominently as a possible reaction product of IAA oxidation. The data on substrate utilization (14) and the respiratory quotients observed (15) were suggestive in this respect. Only recently (9) has this product been detected in small amounts. Earlier studies of the author (13) showed that with the enzyme from *Lupinus albus*, regardless of its state of purity, significant amounts of the aldehyde were not accumulated. Therefore, in order to maintain the role of the aldehyde as an intermediate product of IAA-oxidation, one must assume either its rapid further oxidation in the reaction sequence or that it is not formed in quantity under the experimental conditions. However, the first hypothesis cannot be substantiated since the oxidase obtained from lupine is incapable of utilizing indole-3-carboxaldehyde as a substrate.

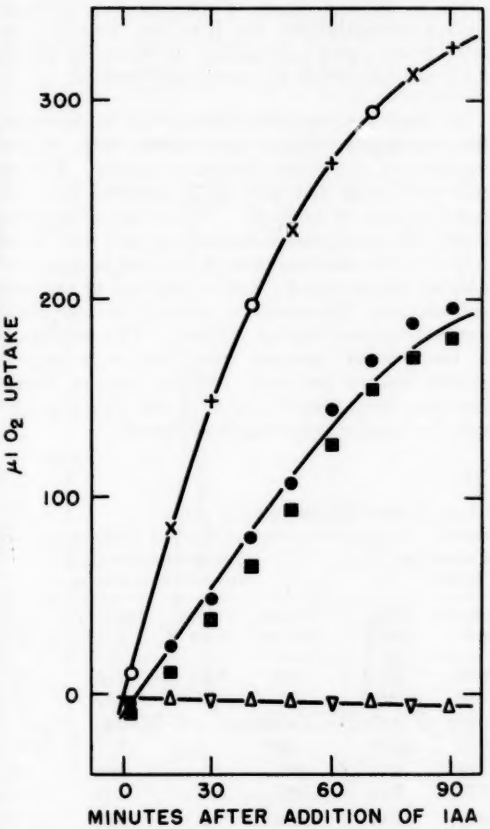

Fig. 1. Effect of cytochrome system on oxygen uptake with a dialyzed oxidase preparation, Mn^{++}, and 2,4-dichlorophenol. ○, reaction mixture without added ingredients; ×, cytochrome oxidase; +, Al^{+++}; △, cytochrome c; ■, cytochrome c + cytochrome oxidase; ●, cytochrome c + cytochrome oxidase + Al^{+++}; ▽, cytochrome system minus IAA oxidase, or IAA oxidase plus the cytochrome system minus 2,4-dichlorophenol.

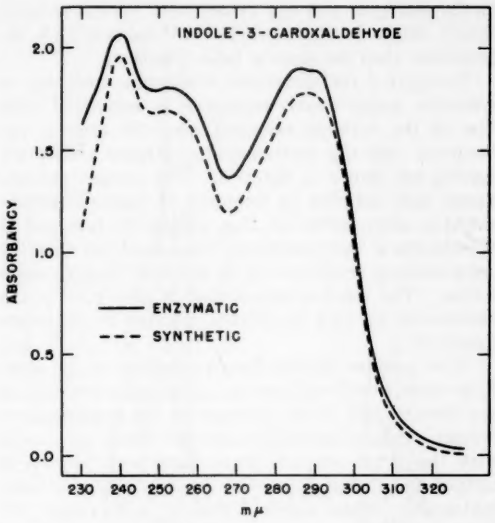

Fig. 2. The ultraviolet absorption spectrum of synthetic indole-3-carboxaldehyde and of material isolated from enzyme reaction mixture, in ethanol.

In the present experiments it was found that, if the right concentration ratios of oxidase and cytochrome c are selected, a 50 % depression in the oxygen uptake is observed as is nearly the case in figure 1. Before the concentration response data in table I were established, this restriction of the oxygen uptake was thought to indicate a complete shift in the metabolic pattern. However, the relationship between oxygen uptake and cytochrome c (table I) may be conservatively interpreted to suggest that cytochrome c is inhibitory up to a concentration of 10^{-6} M, at which level it is slightly stimulatory. The aldehyde response is parallel to the oxygen uptake in part, but the highest level of aldehyde is reached at 10^{-5} M cytochrome c, and the most efficient conversion of IAA to aldehyde is at 5×10^{-5} M cytochrome c. At this level, with 0.5 ml of enzyme, approximately 6 moles of IAA were oxidized by three moles of oxygen to give three moles of aldehyde. This molar ratio would be significant if the Salkowski assay could be assumed to be reliable; however, the 1-ml enzyme experiment (table I) and the C^{14} data (fig 4 and 5) suggest that this is not the case. It is attractive to suggest that the cytochrome system is competing as the terminal oxidase by diverting hydrogen from the peroxidase portion of the oxidase-peroxidase system so that a reducible intermediate is not regenerated.

The relation of the formation of the # 4 product

to the aldehyde and the cytochrome system is more clearly shown in the methylene-C^{14} labeled IAA experiments than the data in table I indicate.

In figure 2 the ultraviolet absorption spectrum of synthetic indole-3-carboxaldehyde is compared with that of the material obtained from the enzyme experiment, and the corresponding infrared absorption spectra are shown in figure 3. The present identification and isolation in quantity of indole-3-carboxaldehyde after action of IAA oxidase in presence of cytochrome c and cytochrome oxidase shows that the usual reaction sequence can be diverted from its main course. The relative significance of this part in the metabolism of IAA by plants remains to be established (5).

Two possible intermediate precursors of the aldehyde were tested with crude and purified enzyme, in the absence and in the presence of the cytochrome c system. Indole-3-glyoxylic acid was totally inert even with the crude enzyme, since there was no oxygen uptake or product formation as shown by the chromatograms, while indole-3-glycolic acid (table II) gave the aldehyde in substantial amounts independent of the cytochrome system. Neither acid shows any oxygen uptake with the cytochrome system in the absence of the oxidase, although a trace of the aldehyde could be detected on the chromatograms from the reaction of indole-3-glycolic acid and the cytochrome system. Recently Greenburg and Galston (4) have observed the instability of indole-3-glycolic acid and have suggested a role for it in the formation of auxin. Indole-3-glyoxylic acid should presumably be the intermediate between indole-3-glycolic acid and the aldehyde; the fact that it was not utilized by IAA oxidase suggests that decarboxylation occurs simultaneously with dehydrogenation.

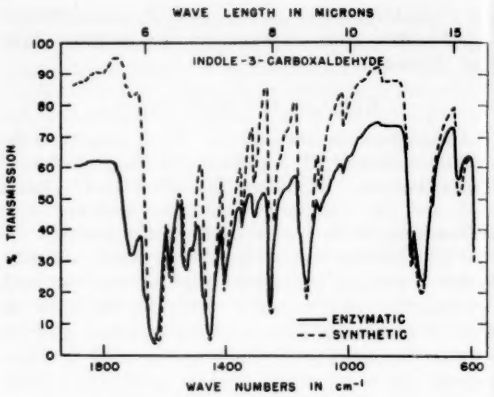

FIG. 3. Infrared spectra of synthetic and enzymatic indole-3-carboxaldehyde; the KBr disc technique was used. In the region not shown in the figure, the spectra of the two compounds were essentially identical.

To shed light on the primary site of oxidation, three substituted IAAs were studied with enzyme preparations of different degrees of purity. The results with crude and with highly purified IAA oxidase are given in table II. Despite the substitution in the side chain, indole-3-isobutyric acid was found to be a better substrate than IAA in that it supported a higher oxygen uptake, and, in the case of the electrophoretically homogeneous enzyme, the chromatograms showed no residual substrate. Chromatograms of the reaction mixture from the crude enzyme showed residual substrate. Similar oxygen uptake data have been reported by Ray and Thimann (10) with the enzyme from *Omphalia flavida*.

TABLE II

RESPONSE OF SUBSTITUTED INDOLE-3-ACETIC ACID TO THE OXIDASE FROM L. ALBUS

SUBSTRATE	CYTOCHROME SYSTEM (2×10^{-5} M CYTOCHROME c)	CRUDE OXIDASE FROM ETIOLATED LUPINE HYPOCOTYLS				ELECTROPHORETICALLY HOMOGENOUS OXIDASE			
		UPTAKE * OXYGEN	IAA USED **	PRODUCT # 4 †	ALDEHYDE ‡	OXYGEN UPTAKE *	IAA USED **	PRODUCT # 4 †	ALDEHYDE ‡
IAA	–	10.6	16	0.69	0.21	15.2	22	1.06	1.03
	+	11.4	16	0.69	3.93	11	16.3	0.67	5.17
Indole-3-glycolic acid	–	6.5	...	0.085	16.1	9.25	...	0.17	24.9
	+	7.2	...	0.12	18.2	8.84	...	0.17	25.9
Indole-3-isobutyric acid	–	16.1	...	1.08	0.21	20.9	...	1.89	1.03
	+	19	...	1.73	0.21	20.3	...	2.91	1.45
1-Methylindole-3-acetic acid	–	11.4	...	0.65	0.41	6.83	...	0.64	0.85
	+	12	...	0.67	3.86	8.0	...	0.69	5.59
2-Carboxyindole-3-acetic acid	–	10	...	0.1	0.0	0.0	...	0.0	0.0
	+	4.3	...	0.0	0.0	0.0	...	0.0	0.0

All values are expressed in micromoles.

* These values were obtained after 90 min at 30° C. The initial substrate concentration was 30 micromoles.

** Salkowski test with IAA as the reference standard.

† The analysis of product # 4 and of indole-3-carboxaldehyde is described in the text.

‡ In the colorimetric determination, indole-3-carboxyaldehyde was used as the reference standard. This restricts the accuracy of the results obtained with the IAA derivatives.

Considering the level of substrate utilized and the oxygen uptake, the amount of substance reacting with 2,4-dinitrophenylhydrazine was low; it is possible that an oxindole (11) causes the response. However, these data suggest that the alpha carbon of the side chain is not the primary site of oxidation. It appears rather that the ring system is the site of attack and that ring substitution should block the oxidation. Two compounds of this type were tested: 1-methylindole-3-acetic acid and 2-carboxylindole-3-acetic acid.

With the purified IAA oxidase, no oxygen uptake was observed with 2-carboxyindole-3-acetic acid as a substrate. The ability of the frozen crude enzyme preparations from 10-day-old etiolated hypocotyls, in contrast to the electrophoretically purified material, to metabolize the 2-carboxy-IAA constitutes the first evidence of a restriction in range introduced by purification of the system. This suggests that part of the changes in activity observed on aging and purification of the crude enzyme from both etiolated and non-etiolated tissue may be caused by the loss of some essential material. The dialyzed enzyme gave results similar to the electrophoretically purified enzyme preparation. In further experiments, none of the lupine enzyme preparations has been found capable of oxidizing 5-hydroxy-IAA, tryptophan, indole-3-acetonitrile, indole-3-carboxaldehyde, or indole-3-carboxylic acid.

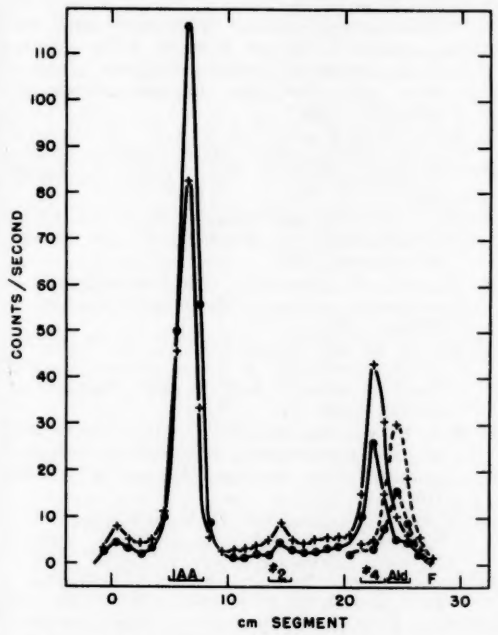

FIG. 4. Paper chromatography of the products from IAA-2-C^{14} obtained by enzymatic reaction in TRIS buffer at pH 7.2. The locations of residual IAA, products # 2, # 4, indole-3-carboxaldehyde, and of the solvent front are indicated. The reaction time was 30 minutes (●), or 90 minutes (+). The solid lines show the response to the IAA oxidase system alone, the broken lines the results with addition of the cytochrome c-cytochrome oxidase system (2×10^{-5} M cytochrome c).

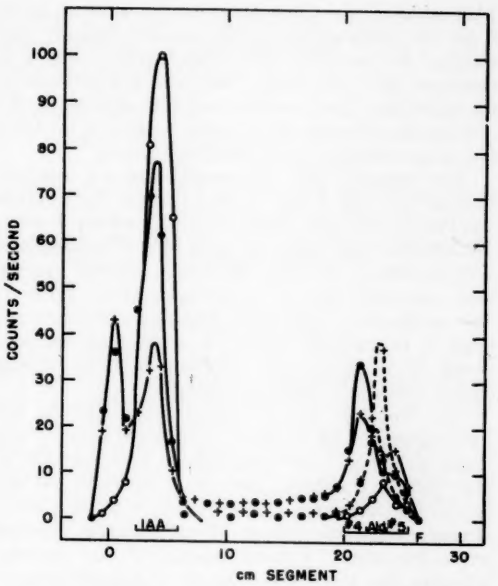

FIG. 5. Paper chromatography of the reaction products from IAA-2-C^{14} obtained in phosphate buffer, pH 6.3. The solid lines show the effect of the oxidase system, the broken lines indicate the results in presence of IAA oxidase and cytochrome system (2×10^{-5} cytochrome c). The reaction times were 30 minutes (●) and 90 minutes (+). Shaking of the IAA for 90 minutes (○) without enzyme proved that little spontaneous decomposition had taken place.

Attempts were made to amplify and corroborate the data on the reaction by experiments with C^{14}-labeled IAA, with the label either in the carboxyl group (IAA-1-C^{14}) or in the methylene group (IAA-2-C^{14}) of the side chain (12, 13).

The concentration of IAA-2-C^{14} declined during the course of the reaction, as measured by the decrease in its C^{14} counts, and increasing activity was recovered in the products. This correlation was observed with samples taken after 30-, 60-, and 90-minute reaction periods (see figs 4 and 5, in which the 60-minute values are omitted). Fig 4 shows the activity distribution found on the chromatograms when the oxidase used IAA-2-C^{14} as a substrate, in the presence and absence of the cytochrome system, in TRIS buffer at pH 7.2. Similarly, figure 5 shows the response to the same enzymes in phosphate buffer at pH 6.3. pH 6.3 is the optimum for the partially purified oxidase used, while the pH 7.2 TRIS buffer represents compromise with the optimum for cytochrome oxidase.

The effect of the cytochrome system (minus the

IAA oxidase) after 90 minutes is also shown in figure 5 by the presence of activity in the aldehyde and product # 5 areas of the chromatogram. The predominant shift in activity from the # 4 product to the aldehyde, which is found on the addition of the cytochrome system in both figures 4 and 5, is evidence of a major change in the reaction pathway.

The unstable and apparently complex reaction product # 4 (fig 4 and 5) may contain the oxindole described by Ray and Thimann (10). However, when this fraction was removed from the chromatograms and examined, the ultraviolet and infrared spectra were complex, and on rechromatography, three or four components were found, including one which appears to be an anthranilate. It is assumed that oxidation or decomposition, or both, occurred during manipulation and rechromatography.

The immobile fraction found below IAA on the chromatogram (fig 5) could be detected only by the tracer technique; it is neither fluorescent nor does it show indolyl reactions. Phosphate buffer at pH 6.3 favors its formation. The presence of the cytochrome-cytochrome oxidase system had little influence on its formation. Infrared spectra of this material indicated the absence of phenyl or indolyl groups; the only discernible functional groups were methylene and carboxyl groups. The aliphatic nature of this reaction product is thus indicated, and because of the immobility on chromatograms, a strongly acid character is suggested.

Experiments with carboxy-C^{14}-IAA yielded predominantly non-radioactive products; the C^{14} was recovered as $BaC^{14}O_3$ from the center well. An exception to this was the non-indolyl product # 2 formed in TRIS buffer (fig 4), which still carried C^{14}, whether carboxyl- or methylene-labeled IAA was used as a substrate. It is weakly acidic, shows green fluorescence, and is assumed to be aromatic. The buffer used for its formation is immaterial.

The present data show that catabolism of IAA may occur by more than one route. With respect to the primary site of action of IAA oxidase, the observations are in harmony with the results of other laboratories (7, 10, 11); the ring system is altered by the primary attack. Further work will be required to unravel the reaction sequence and identity of the products.

The author wishes to acknowledge the assistance of Mrs. Sara Berliner in carrying out many of these experiments.

Summary

Indole-3-carboxaldehyde is a major reaction product of the action of IAA oxidase provided the system is coupled to the cytochrome-cytochrome oxidase system. Indole-3-glycolic acid yields indole-3-carboxaldehyde with equal efficiency both in the absence and presence of cytochrome c. Experiments with substituted indole-3-acetic acids as substrate indicate that the ring system, not the side chain, is the primary site of oxidation. The two position of the indole ring is probably the primary site of dehydrogenation. The use of IAA-2-C^{14} as substrate made possible the detection of a reaction product which apparently is an aliphatic acid. The formation of this product is unaffected by the cytochrome system suggesting that it is the terminal oxidation product.

Literature Cited

1. Elks, J., Elliott, D. F. and Hems, B. A. Studies in the indole series. Part III. Improved synthesis of tryptophan. Jour. Chem. Soc. 629–632. 1944.
2. Erdtman, H. and Jönsson, Å. Synthetic plant hormones. V. Synthesis of alpha-3-indoleisobutyric acid, an antagonist of indole-3-acetic acid, and of some other 1- and 3-indole acids. Acta Chemica Scand. 8: 119–126. 1954.
3. Gordon, S. A. and Weber, R. P. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26: 192–195. 1951.
4. Greenburg, J. B. and Galston, A. W. Formation and auxin activity of indole-3-glycolic acid. Science 125: 992–993. 1957.
5. Jones, E. R. H. and Taylor, W. C. Some indole constituents of cabbage. Nature 179: 1138. 1957.
6. Maclachlan, G. A. and Waygood, E. R. Kinetics of the enzymically-catalyzed oxidation of indoleacetic acid. Can. Jour. Biochem. Physiol. 34: 1233–1250. 1956.
7. Manning, D. T. and Galston, A. W. On the nature of the enzymatically catalyzed oxidation products of indoleacetic acid. Plant Physiol. 30: 225–231. 1955.
8. Platt, R. S., Jr. and Thimann, K. V. Interference in Salkowski assay of indoleacetic acid. Science 123: 105–106. 1955.
9. Racusen, D. Formation of indole-3-aldehyde by indoleacetic oxidase. Arch. Biochem. Biophys. 58: 508–509. 1955.
10. Ray, P. M. and Thimann, K. V. The destruction of indoleacetic acid. I. Action of an enzyme from *Omphalia flavida*. Arch. Biochem. Biophys. 64: 175–192. 1956.
11. Ray, P. M. The destruction of indoleacetic acid. II. Spectrophotometric study of the enzymatic reaction. Arch. Biochem. Biophys. 64: 193–216. 1956.
12. Stutz, R. E., Atkinson, D. E. and Gordon, S. A. Synthesis of 3-indoleacetic acid-2-C^{14}. Argonne National Laboratory Report ANL-4710. 1951.
13. Stutz, R. E. The indole-3-acetic acid oxidase of *Lupinus albus* L. Plant Physiol. 32: 31–39. 1957.
14. Tang, Y. U. and Bonner, J. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25. 1947.
15. Wagenknecht, A. C. and Burris, R. H. Indoleacetic acid inactivating enzymes from bean roots and pea seedlings. Arch. Biochem. 25: 30–53. 1950.

TRANSLOCATION OF ORGANIC SUBSTANCES IN TREES. III. THE REMOVAL OF SUGARS FROM THE SIEVE TUBES IN THE WHITE ASH (FRAXINUS AMERICANA L.) [1]

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It has been shown by various investigators that the substances in the sieve tubes are qualitatively the same throughout the plant. This, together with a number of physiological observations, seems to suggest that substances move through the sieve tubes without their being metabolized (2, 8, 23). There is good evidence, however, that the movement over the short distance from the leaf parenchyma to the sieve tubes is an active process. It has been shown by Phillis and Mason (13) that the sugar concentration of sap expressed from leaf tissue containing mainly mesophyll is much lower than that of sap expressed from the inner part of the bark or of leaf tissue containing mainly veins. This was later confirmed by a number of other workers using a wide variety of plants and different methods. Most important—since concentrations of expressed saps are not directly comparable—is the fact that leaf parenchyma cells have been plasmolyzed by the sieve tube exudate of the same plant (7, 14). Transfer of substances into the sieve tubes must therefore occur metabolically. This process has been called "loading" by Barrier and Loomis (1). Huber and Bauer refer to it as "secretion" (9) because of the striking physiological similarity to the secretion of sugars in nectaries (10, 20, 21). There are a few reports on this movement dealing with both the chemistry of the transferred material and the physiology of the tissues involved (2, 18, 21), but the details are far from known.

A parallel problem to that of the introduction of assimilates into the sieve tubes is that of how the assimilates are removed from these translocation channels. The mere fact that they do leave the sieve tubes has led some investigators to believe that the sieve tubes are "leaking." That this is not the case is indicated by the fact that they are, even at great distance from the leaves, under a high turgor pressure most of the time (see (5)). It will be shown in this paper that the turgor remains high for many days after the leaves have formed abscission layers in the fall, and also after artificial defoliation.

The presence of large amounts of stachyose, raffinose and sucrose make white ash a very interesting object for phloem translocation studies. It is significant that these sugars contain the sucrose unit within their molecules. In order to explain our previous observations it was proposed that there is an enzymatic removal of D-galactose units which results in a gradual break-down of the higher oligosaccharides, stachyose and raffinose, as they move down the tree (24). In the present investigation three ash trees have been defoliated, thus cutting off the supply of photosynthates to the phloem, and the changes that took place in the sieve tubes after this treatment have been followed. A similar study has been made of the changes in the phloem during the natural leaf-fall in autumn.

[1] Received December 30, 1957.

EXPERIMENTAL

The defoliation experiments were carried out in Tom Swamp Tract I of the Harvard Forest. Three ash trees were chosen which had over 9 meters clear trunk length and a diameter at breast height of about 15 cm. On July 13, 1957, they were completely defoliated with the aid of a "sky-worker." Defoliation of one tree required about 40 minutes. Samples of

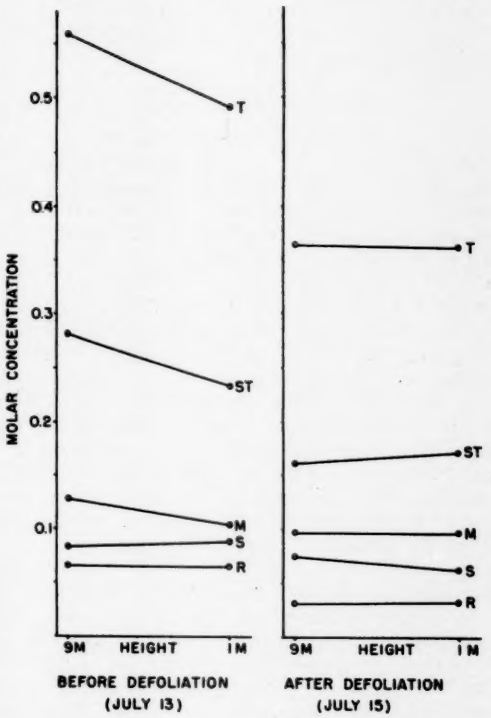

FIG. 1. Gradients along the tree trunk before and after defoliation. The four substances shown represent about 90% of the exudate's dry weight. As soon as the leaves are removed, the total concentration drops, the total molar concentration gradient disappears and some of the individual gradients (stachyose and to a slight extent raffinose) become negative. M = mannitol, S = sucrose, R = raffinose, ST = stachyose, T = total conc.

sieve tube exudate were taken on the day before and on several successive days after defoliation. Four tapping cuts were applied each time at 1 and 9 meters height from the ground. From each cut two 5-μl pipettes (for sugar analysis), three 1-μl and three 2-μl pipettes (for mannitol analysis) were filled. Each point on figure 1 represents therefore the average of 8 sugar or 24 mannitol determinations. During the natural leaf-fall, samples were collected from two further trees in much the same way.

The samples were brought to the laboratory, transferred onto the paper chromatograms and dried. The whole process was completed within about one hour from the time of collection. The chromatograms were then stored in a freezer at $-20°$ C, wrapped in polyethylene foil in packages containing the analytical work of one day (64 sugar analyses including 16 standard values). The analysis of the samples was carried out as previously described (22, 23).

There were no significant enzymatic interconversions after collection. Several days elapsed before a sample, stored at room temperature, showed the first fermentation products (reducing sugars). Such products were not detected during chromatographic analysis following proper storage.

Results and Discussion

It was expected, according to observations that had been made in the previous autumn during leaf-fall, that the sap-flow would last for only a few days after defoliation. Surprisingly, however, sieve tube exudate could always be obtained, and samples were taken for about a month until the trees returned to normal activity with their newly formed leaves. The results from all three trees were the same.

The quantitatively most important substances in the sieve tube exudate of white ash are stachyose, raffinose, sucrose, and D-mannitol (23). They constitute about 90 % of the dry weight of the exudate. There is therefore little error in calculating osmotic pressures from the molar concentrations of these four substances. The gradients before and after defoliation are shown in figure 1. The total concentration drops in the whole sieve tube system immediately after the assimilate supply is cut off. The total molar concentration gradient, which is positive in the downward direction of the trunk throughout the whole summer, disappears. At the same time, and probably as a result of this, some of the gradients of individual substances become negative. This confirms the previously reported observation made after the leaf-fall in autumn (23). It supports the supposition that the turgor gradient is the driving force of translocation through the sieve tubes.

One way of studying the exit of sugars from the sieve tubes would be to isolate a short piece of trunk and to analyze its sieve tube content continuously. Instead of doing this, the samples can just as well be taken from a defoliated tree since defoliation stops the supply of photosynthates to the phloem. Samples taken from the middle of the entire phloem

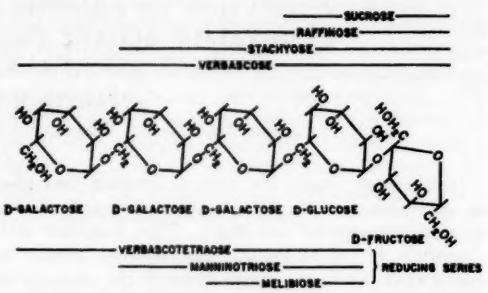

Fig. 2. The raffinose family of oligosaccharides. The sugars of the sieve tube exudate of white ash are sucrose, raffinose, stachyose and verbascose. Verbascose is present in traces only and therefore disregarded in this quantitative study. The reducing series are not native sugars in the exudate, but occur only as fermentation products. (From Zimmermann (24).)

length will yield average values, since gradients are fairly linear. However, in order to obtain gradients from the same experiments samples were taken at 1 and 9 meters height, and the values corresponding to the 5 meter level were calculated by averaging the 1 and 9 meter values. Figure 3 shows the change in sugar concentrations after defoliation. During the 1st 8 days stachyose rapidly decreases. Sucrose increases, obviously at the expense of stachyose. It will be seen from the relationship of the sugars, which is shown in figure 2, that the enzyme capable of such a transformation is an α-D-galactosidase. Since the galactose units never appear in the exudate, it follows that they must be transferred directly out of the sieve tube vacuoles.

The mode of action of such a galactose removal system may be outlined as follows: the enzyme is attached to the inner surface of the side-wall cytoplasm of the sieve tubes. It acts upon the oligosaccharides while they pass, removing D-galactose units and thus producing the next lower oligosaccharide. So long as the supply continues this break-down only shows as a gradient in the concentrations of the oligosaccharides (cf. fig 1). However, as soon as the oligosaccharide supply from above is cut off by defoliation, the break-down becomes apparent. Evidently, it proceeds almost to completion.

There must be a mechanism for the removal of sucrose but we know little about it. This process is very important, since in many plants sucrose is the only translocatory sugar (11, 16, 19, 21, 22). The sucrose curve of figure 3 does not indicate sucrose removal, since, at the same time, there is a sucrose production through break-down of the higher oligosaccharides. However, the removal of sucrose molecules from the sieve tubes is shown by the decrease of the total molar sugar concentration, because transgalactosidation does not affect the total molarity. In other words, the total sugar molarity actually represents the total sucrose molarity, although part of the sucrose units are masked as raffinose and

stachyose. Thus a measure of the rate at which the sucrose molecules leave the sieve tubes is given by the total sugar molarity curve in figure 3. This curve shows that the sucrose removal is very rapid immediately after defoliation, slows down, and, after the 8th day even becomes reversed, that is, the sucrose content increases somewhat without a corresponding break-down of higher oligosaccharides. This rapidly decreasing sucrose removal rate might be explained in the following way: the sucrose removal is somehow controlled by a hormonal factor that comes from the leaves with the assimilate stream. As soon as the tree is defoliated the hormone is exhausted and the removal process stops. It may even become reversed, that is, sucrose may reenter the sieve tubes (after the 8th day in our experiment). It might be argued that the rapid concentration drop is actually some kind of a dilution effect, due to the sudden increase of the relative pressure within the xylem after defoliation. This is hardly possible, since it has been shown that the concentration does not drop at night when transpiration is low (8, 21, 24). Moreover, the rapid exit of sucrose immediately after defoliation must be the same as in a normal, undisturbed tree if we consider the length of the phloem and the order of magnitude of translocation speeds that have been found by many investigators (3, 4, 6, 12, 15, 17).

It will be seen in figure 3 that both the rate of sucrose disappearance—as pictured by the total sugar molarity curve—and the rate of break-down of stachyose drop after defoliation, although the latter drops slowly. In the experiment shown in figure 3 the break-down rate of stachyose is 1.31 % per hour during the 1st day after defoliation, 0.91 % per hour during the 2nd day, and 0.59 % per hour during the 2nd to 8th day. In other experiments it was: 0 to 1st day = 1.44 %/hr., 1st to 4th day = 1.00 %/hr., 4th to 10th day = 0.47 %/hr.; and 1st day = 1.39 %/hr., and 1st to 5th day = 0.61 %/hr. This decrease in rate of stachyose break-down may be due to a decreased enzyme activity, i.e., may be proportional to the lowered substrate concentration, or it may be due to a decreased mechanical turnover of the vacuole content when the flow slows down and finally stops. In other words, fewer stachyose molecules would make contact with the enzyme on the inner cytoplasm surface of the sieve tubes when the assimilate stream becomes stagnant.

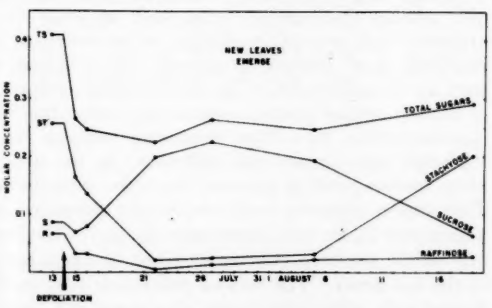

FIG. 3. The effect of defoliation on the sieve tube sugar concentrations at 5 meters height, the approximate middle of the entire phloem length.

The rate of stachyose break-down is probably nearest to normal immediately after defoliation. This allows us to make an approximate calculation of the rate of translocation of stachyose on the day before defoliation. The stachyose decreased then from 0.281 M at 9 meters to 0.233 M at 1 meter height, which is a decrease of 17 % over a distance of 8 meters. During the first 27.5 hours after defoliation the stachyose decrease at 5 meters height, which represents about the average of the whole tree, was 1.31 % per hour. If we consider this break-down rate normal, the decrease of 17 % would have required 17/1.31 or 13 hours, and must be the time taken to travel 8 meters. This corresponds to a stachyose translocation rate of 62 cm per hour. The same calculation carried out with the data of the two other experiments yields speeds of 73 and 54 cm per hour respectively. These figures are of the order of magnitude of translocation rates that have been found by many other investigators using different methods (for instance 3, 4, 6, 12, 17). It would be interesting to compare the rates of all the different substances that are present in the sieve tubes. Unfortunately, however, stachyose is the only substance that allows the application of such calculations, because we are reasonably sure it is the only one that fulfils the requirements of being synthesized only in the leaves and broken down only in the sieve tubes (cf. discussion in (24)). Raffinose and sucrose are also synthesized in the leaves but they are produced in the sieve tubes by galactose removal from higher oligosaccharides while they move down the trunk, and there are indications that D-mannitol is also produced in the sieve tubes, possibly in connection with the sucrose exit.

Production of sucrose through oligosaccharide break-down would explain why, in a few instances, the sucrose gradient—and only the sucrose gradient—in undisturbed trees was found to be slightly negative in the downward direction during the summer. Figure 1 left, which shows the gradients before defoliation, presents an example of this. The rate of sucrose production by oligosaccharide break-down may sometimes exceed the rate of sucrose removal.

New leaves emerged on all defoliated trees after about two weeks. At this time (July 27, fig 3) the increase in sugar concentration changes to a decrease. This shows that the growing leaves draw upon the sugar supply of the phloem. The final increase after August 8, and particularly the reappearance of stachyose indicate that newly formed leaves are exporting photosynthates again.

The situation during the natural leaf-fall in autumn is quite similar to that of artificial defoliation. Figure 4 shows the data obtained from a tree between September 26 and October 21, 1957. If these curves are compared with those of figure 3 it will be seen

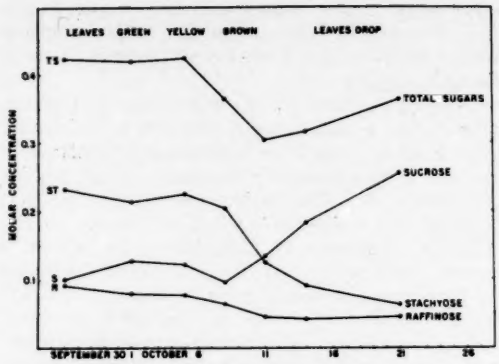

FIG. 4. Sieve tube sugar concentrations during the natural leaf-fall in autumn. A comparison of these curves with those of figure 3 suggest that the abscission layer was formed between October 5 and 8, a few days after the leaves turned yellow.

that the point equivalent to the time of defoliation must fall on about October 5 to 7. Obviously the abscission layer was then formed. If samples are taken around the trunk at one height in late autumn, it may be found that the sugar conversion is farther advanced on one side of the trunk than on another. This observation, which has been previously reported (cf. (24) figure 6), may be due to an earlier formation of the abscission layer on parts of the crown. The changes in sugar concentrations after October 5 suggest that the leaves, although still on the tree, were physiologically disconnected from the phloem of the trunk. The changes in sugar concentrations between October 5 and 21 are the same as those between July 14 and 27 of the defoliation experiments.

The sucrose increase between October 11 and 21 (fig 4) and between July 22 and 27 (fig 3) is of interest. This increase occurs during a time when the concentration of the higher oligosaccharides is low and does not change significantly (fig 3). It is therefore not due to oligosaccharide break-down. Nor is it due to a random variation in samples, because it appeared invariably in all experiments and the maximum variation of the individual samples did not exceed $\pm 10\%$ (including a chromatographic error of ± 2 to 4%). The increase in sucrose may be nothing more than a mild desiccation of the phloem tissue due to continued removal of outer bark, which is necessary for tapping. The hormonal exit control that has been discussed above may also explain the sucrose increase. Sucrose exit may be a reversible process, going in one direction in the presence of the hormonal factor (sucrose exit) and in the other direction in the absence of it (sucrose re-entry). As soon as the tree is defoliated, sucrose exit slows down, stops, and becomes reversed (sucrose re-entry—due to the exhaustion of the hormone). The translocates in the sieve tubes, instead of being depleted, are thus saved. This hypothesis, if correct, explains how trees that have been defoliated by natural forces such as hail or insects can immediately produce new leaves from reserve material.

The last samples were taken on October 21; no more exudate could be obtained after this time. The sap flow was poor on October 21 in spite of the relatively high concentrations. This suggests that it is not a decreasing sieve tube turgor caused by decreasing concentrations that stops the sap flow in late autumn, but rather something else, possibly callus formation or active sieve tube shrinkage (cf. discussions in (8) and (24)). We made no attempt to check this anatomically.

These experiments are consistent with results reported previously (23, 24), which suggests that there is a passive flow of solutes along a positive turgor gradient which is established and maintained by the metabolic entry of solutes into and removal from the sieve tubes. The secretion of photosynthates into the sieve tubes in the leaves, discussed by a number of workers (1, 2, 21), may be regarded as a metabolic "pressure-pump." It raises the turgor within the sieve tubes of the leaves. The removal mechanisms, which lower the sieve tube turgor at places of solute consumption, would then represent a metabolic "suction-pump," located at the other end of the translocation channel, working under remote control from the leaves.

SUMMARY

The sieve tube sugars of white ash (*Fraxinus americana* L.) were analyzed after artificial defoliation in summer and during the natural leaf-fall in autumn. After artificial defoliation the sieve tubes remained turgescent for an unlimited time. New leaves emerged after approximately two weeks. In autumn the sieve tubes could be tapped until two to three weeks after the abscission layer had been formed. Since sieve tubes retain their turgescence for such a long time after the interruption of the assimilate supply, it is concluded that they are not "leaking." Two metabolic mechanisms are described, both of which may be responsible for the removal of sugars from the sieve tubes.

In the first place there is a rapid interconversion of stachyose into sucrose. This was observed after artificial and natural defoliation as a decrease in stachyose and increase in sucrose. It is suggested that an α-D-galactosidase on the side-wall cytoplasm of the sieve tubes removes D-galactose units while the oligosaccharides pass, thus producing raffinose from stachyose and sucrose from raffinose. In the second place sucrose itself is removed from the sieve tubes. This process seems to be reversible and controlled by a hormonal factor that comes from the leaves (sucrose exit in the presence, sucrose re-entry in the absence of the hormone). The sucrose removal is very rapid immediately after defoliation (hormone still available), but slows down and stops after five days, after which sucrose increases again in the sieve tubes (hormone exhausted).

From the break-down rate of stachyose immediately after defoliation, and the measured stachyose gradient along the trunk before defoliation, it was possible to calculate the rate of stachyose translocation as 62, 73, and 54 cm per hour for the three defoliation experiments.

The total molar gradient of the three sugars and D-mannitol, which constitute about 90 % of the sieve tube substances, is positive in the downward direction of the trunk during the whole summer. After defoliation this gradient disappears and at the same time some of the gradients of the individual substances become negative. This suggests that the turgor pressure gradient is the driving force of translocation through the sieve tubes.

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Literature Cited

1. Barrier, G. E. and Loomis, W. E. Absorption and translocation of 2,4-dichlorophenoxyacetic acid and P^{32} by leaves. Plant Physiol. 32: 225–231. 1957.
2. Bauer, L. Zur Frage der Stoffbewegungen in der Pflanze mit besonderer Berücksichtigung der Wanderung von Fluorochromen. Planta 42: 267–451. 1953.
3. Biddulph, O. and Markle, J. Translocation of radiophosphorus in the phloem of the cotton plant. Amer. Jour. Bot. 31: 65–70. 1944.
4. Crafts, A. S. Sieve-tube structure and translocation in the potato. Plant Physiol. 8: 81–104. 1933.
5. Crafts, A. S. Movements of assimilates, viruses, growth regulators, and chemical indicators in plants. Bot. Rev. 17: 203–284. 1951.
6. Crafts, A. S. and Lorenz, O. A. Fruit growth and food transport in cucurbits. Plant Physiol. 19: 131–138. 1944.
7. Curtis, O. F. and Asai, G. N. Evidence relative to the supposed permeability of sieve tube cytoplasm. Amer. Jour. Bot. 26: 16s–17s. 1939.
8. Huber, B. Anatomical and physiological investigations on food translocation in trees. In: The physiology of forest trees; Cabot Foundation Symposium on Tree Physiology, K. V. Thimann, ed. Ronald Press, New York 1958.
9. Huber, B. and Bauer, L. Wasserumsatz und Stoffbewegungen. Fortschr. Bot. 18: 227–241. 1956.
10. Matile, Ph. Ueber den Stoffwechsel und die Auxinabhängigkeit der Nektarsekretion. Ber. schweiz. bot. Ges. 66: 237–266. 1956.
11. Mittler, T. E. The phloem sap supply to aphids feeding on willow trees. In: The physiology of forest trees; Cabot Foundation Symposium on Tree Physiology, K. V. Thimann, ed. Ronald Press, New York 1958.
12. Münch, E. Versuche über den Saftkreislauf. Ber. deut. bot. Ges. 45: 340–356. 1927.
13. Phillis, E. and Mason, T. G. Studies on the transport of carbohydrates in the cotton plant. III. The polar distribution of sugar in the foliage leaf. Ann. Bot. 47: 585–634. 1933.
14. Röckl, B. Nachweis eines Konzentrationshubes zwischen Palisadenzellen und Siebröhren. Planta 36: 530–550. 1949.
15. Schumacher, W. Untersuchungen über die Wanderung des Fluoresceins in den Siebröhren. Jahrb. wiss. Bot. 77: 685–732. 1933.
16. Swanson, C. A. and El-Shishiny, E. D. H. Translocation of sugars in the Concord grape. Plant Physiol. 33: 33–37. 1958.
17. Vernon, L. P. and Aronoff, S. Metabolism of soybean leaves. IV. Translocation from soybean leaves. Arch. Biochem. Biophys. 36: 383–398. 1952.
18. Wanner, H. Phosphataseverteilung und Kohlenhydrattransport in der Pflanze. Planta 41: 190–194. 1952.
19. Wanner, H. Die Zusammensetzung des Siebröhrensaftes: Kohlenhydrate. Ber. schweiz. bot. ges. 63: 162–168. 1953.
20. Ziegler, H. Phosphataseaktivität und Sauerstoffverbrauch des Nektariums von Abutilon striatum Dicks. Naturwiss. 42: 259–260. 1955.
21. Ziegler, H. Untersuchungen über die Leitung und Sekretion der Assimilate. Planta 47: 447–500. 1956.
22. Zimmermann, M. H. Translocation of organic substances in trees. I. The nature of sugars in the sieve tube exudate of trees. Plant Physiol. 32: 288–291. 1957.
23. Zimmermann, M. H. Translocation of organic substances in trees. II. On the translocation mechanism in the phloem of white ash (*Fraxinus americana* L.). Plant Physiol. 32: 399–404. 1957.
24. Zimmermann, M. H. Translocation of organic substances in the phloem of trees. In: The physiology of forest trees; Cabot Foundation Symposium on Tree Physiology, K. V. Thimann, ed. Ronald Press, New York 1958.

FAT UTILIZATION AND COMPOSITION IN GERMINATING COTTON SEEDS[1,2]

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Diminution of the fat reserve and concomitant increase of carbohydrate during germination of oil seeds have been well established (3, 7, 10, 11, 12, 13, 16). Few studies, however, have compared illuminated and unilluminated seedlings, or have controlled the environmental conditions to the degree necessary for calculation of a rate constant and for prediction of fat content at various stages of growth. MacLachlan (9) found a greater utilization of fat during soybean germination in the light than in the dark. He concluded that there was no preferential utilization of unsaturated fatty acids, because no change was observed in the average unsaturation of the fatty acids in the cotyledons. But Holman (6), also using soybeans, reported a decrease in the iodine value of the fat reserve and a preferential utilization of linoleic and linolenic acid. Crombie and Comber (2) found that during the germination of seeds of the watermelon (*Citrullus vulgaris*) all the major fatty acids, except oleic, disappeared at rates proportional to the quantities present in the seed fat. Oleic acid was metabolized relatively faster than the other fatty acids.

A better understanding of the relations of fats to general metabolism may be expected to result from quantitative physiological and chemical studies of germinating fatty seeds. The extent to which reserve fats and current photosynthate contribute to the material and energy requirements for seedling growth represents one aspect of the broad problem. There is also a need for more analytical data on the changes that occur in the fatty acid composition of the glycerides as the fat reserve is utilized.

The experiments reported in this paper were undertaken to study the rate of utilization and the composition of the fat reserve during cotton seed germination under various growth conditions. To correlate the decrease in fat content with the growth of the seedlings, measurements were made of the development of the root, hypocotyl, and cotyledon.

MATERIALS AND METHODS

Cotton seeds of the 1953 crop (*Gossypium hirsutum* L., var. Fox, kindly supplied by the Delta and Pine Land Co. of Scott, Miss.) were reduced in moisture to 6 % by drying with calcium oxide and then stored in sealed metal containers at 1° C (18).

Small lots of seed were delinted in sulfuric acid for three minutes, rinsed thoroughly, soaked for 30 minutes in distilled water, and then allowed to germinate in the dark at 30 ± 1° C on wet filter paper. After 22 hours the seedlings with roots 7 to 12 mm long were supported on a layer of bobbinet (cloth netting with hexagonal mesh 3 by 3 mm) stretched over a glass rod frame provided with 4-cm legs. The frame was placed in a Pyrex dish which was filled to the level of the bobbinet with a culture solution having the following composition: 0.0040 M $Ca(NO_3)_2 \cdot 4\,H_2O$, 0.0030 M KNO_3, 0.0016 M $MgSO_4 \cdot 7\,H_2O$, 0.0012 M KH_2PO_4, 0.0005 M $(NH_4)_2SO_4$, 0.00004 M $FeCl_2 \cdot 4\,H_2O$, 0.00002 M $K_3C_6H_5O_7 \cdot H_2O$ (potassium citrate), and the following amounts in parts per million, of the microtrophic elements: 1.00 B, 0.50 Mn, 0.10 Zn, 0.02 Cu, and 0.02 Mo; the resulting pH was 4.8. The solution was renewed every three days.

The cultures were put in the particular evironment under study, and the testas were slipped off 3.5 hours later. For the experiments in the light the cultures were placed on a rotating table (20). Concentric daylight fluorescent tubes and a Mazda bulb provided a light intensity of 5300 meter candles at the surface of the bobbinet and 6700 meter candles at the level of the cotyledons of plants that had grown for 7 days.

At suitable time intervals the plants were dissected into cotyledons, hypocotyls, and roots. The cotyledons were dropped in boiling water for one minute to arrest enzyme activity. They were dried at 45° C to a moisture content of 5 %, and the remaining water was removed at 55° C in a vacuum oven. The dried cotyledons were passed through a 20-mesh screen in a Wiley mill, extracted (Soxhlet) with petroleum ether (b.p. 30 to 60° C) for 24 hours, ground to fine particles with a mortar and pestle, and then re-extracted with petroleum ether during a 2nd 24-hour period. After the solvent had been removed in a vacuum oven at 40° C, the crude lipide was treated in the following manner: The neutral oil and the unsaponifiable matter were separated from the acidic components by the AOCS Method G-540 (1). Then the alkaline 50 % ethanol solution that contained the latter compounds was acidified, and the ethanol was removed by vacuum distillation at 50° C. The residual solution was extracted with petroleum ether, and the original acidic components were weighed after removal of the solvent. Following saponification of the neutral oil, the unsaponifiable matter was removed by the AOCS Method Ca 6b-40, and the soaps were converted to the acids. The mixed fatty acids were removed from the aqueous layer with petroleum

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[2] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University.

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ether, dried with anhydrous sodium sulfate, and weighed after the solvent had been removed at 40° C in a vacuum oven.

The composition of the mixed fatty acids was ascertained as follows: Triplicate determinations of the iodine value were made by means of the Wijs 30-minute procedure outlined in the AOCS Method Cd 1-25. Two measurements of the percentage of linoleic acid were made by the ultraviolet spectrophotometric method for polyunsaturated acids given in the AOCS Method Cd 7-48. The percentages of oleic acid and of the saturated acids in the sample were calculated by the formulas:

$$\% \text{ oleic acid} = \frac{\text{Iodine value} - (1.811 \times \% \text{ linoleic acid})}{0.899}$$

$$\% \text{ saturated acids} = 100\ \% - \% \text{ linoleic acid} - \% \text{ oleic acid.}$$

The molecular weights of palmitic and oleic acid were used in calculations involving molar quantities, because these two acids make up by far the greatest proportion of the saturated and the monoethenoid acids, respectively (5).

Sufficient numbers of seedlings were grown each time to yield approximately 1 g of crude lipide for analysis. For each stage of development at 30° C, the entire procedure was repeated. But in the experiments that involved the use of temperatures other than 30° C, the procedure was performed only once.

Experiments and Results

Growth of Seedlings at 30° C: During one day on the filter paper the seeds imbibed 21 times their original amount of water. After the seedlings had been supported over the culture solution, water uptake by the cotyledons continued 0.5 day longer and then abruptly ceased (fig 1 A). In the dark, water uptake was not resumed; but in the light, after stopping for one day, water uptake began again and continued at a nearly uniform rate of 18 g per 100 seedlings per day to the end of the 7-day experiment. The 7-day-old cotyledons in the light were dark green, expanded, leaf-like organs, whereas the corresponding ones in the dark were yellow and had not increased beyond the size they had attained in the first 1.5 days.

The combined water content of the hypocotyls and cotyledons (fig 1 B) increased at nearly the same rate in the dark as in the light for the first 4.5 days. In the dark this increase was largely in the hypocotyls; in the light it was chiefly in the cotyledons.

The dry weight of the cotyledons (fig 1 C) decreased continuously in the dark, and by the end of 7 days it had been reduced to about one third of its original value. In the light, however, the decrease soon became less rapid, and after four days the trend was reversed and the dry matter began to increase.

Loss in dry weight of the entire seedling was striking in the initial stages of germination (fig 1 D). During the 1st day following the soaking of the dormant seeds, the initial respiratory loss amounted to 13.3 % of the original dry matter. After the 2nd day, however, a gain in dry weight began, both in the dark and in the light. The seedlings in the dark did not continue to increase in dry weight beyond the 4th day.

Minor Lipide Components: Although, when expressed on a percentage basis, the free fatty acids rose during germination from 0.5 % to 20 %, the actual amount present in 100 seedlings increased only two to three fold (table I).

Moreover, the following data indicate that free fatty acids did not accumulate when growth and fat utilization were retarded. One group of seedlings

Table I

Minor Components of the Crude Lipide *

Age of seedling Days	Acidic matter in lipide		Free fatty acids		Percent free fatty acids **		Unsaponifiable matter	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
0	25	25	12	12	0.6	0.6	22	22
1	39	39	10	10	0.6	0.6	19	19
1.5	52	38	11	10	0.8	0.7	24	24
2	53	76	17	27	1.5	2.2	37	34
2.5	75	70	18	20	1.9	2.5	34	36
3	78	103	15	21	2.3	3.8	38	34
3.5	87	106	22	28	4.5	7.3	29	38
4	94	90	18	39	4.9	13.9	23	40
5	83	86	20	34	9.7	22.1	23	39
7	46	..	18	..	20.5	...	19	..

* Absolute values represent mg per 200 cotyledons (100 plants).

** $\frac{\text{Free fatty acids}}{\text{Fat} + \text{Free fatty acids}} \times 100.$

Table II

Amount of Fat at Various Stages of Development in the Dark and in the Light at 30° C

Age of seedling Days	Fat, % dry weight of cotyledons		Fat content, g per 200 cotyledons		Percentage of seed fat utilized		Percentage of fatty acids in glycerides	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
0	35.1	35.1	1.919	1.919	0	0	92.5	92.5
1	36.7	36.7	1.689	1.689	12.0	12.0	93.6	93.6
1.5	33.3	35.3	1.414	1.523	26.3	20.6	92.6	93.1
2	29.1	28.1	1.153	1.181	39.9	38.5	94.3	92.8
2.5	25.5	20.0	.910	.765	52.6	60.1	92.7	92.3
3	19.5	14.0	.647	.527	66.3	72.5	90.9	...
3.5	15.3	9.5	.465	.359	75.8	81.3	94.2	...
4	12.7	6.6	.347	.243	81.9	87.3	92.2	...
5	7.9	2.9	.187	.120	90.3	93.7	90.4	...
7	3.4	2.0	.070	.100	96.4	94.8	90.9	...

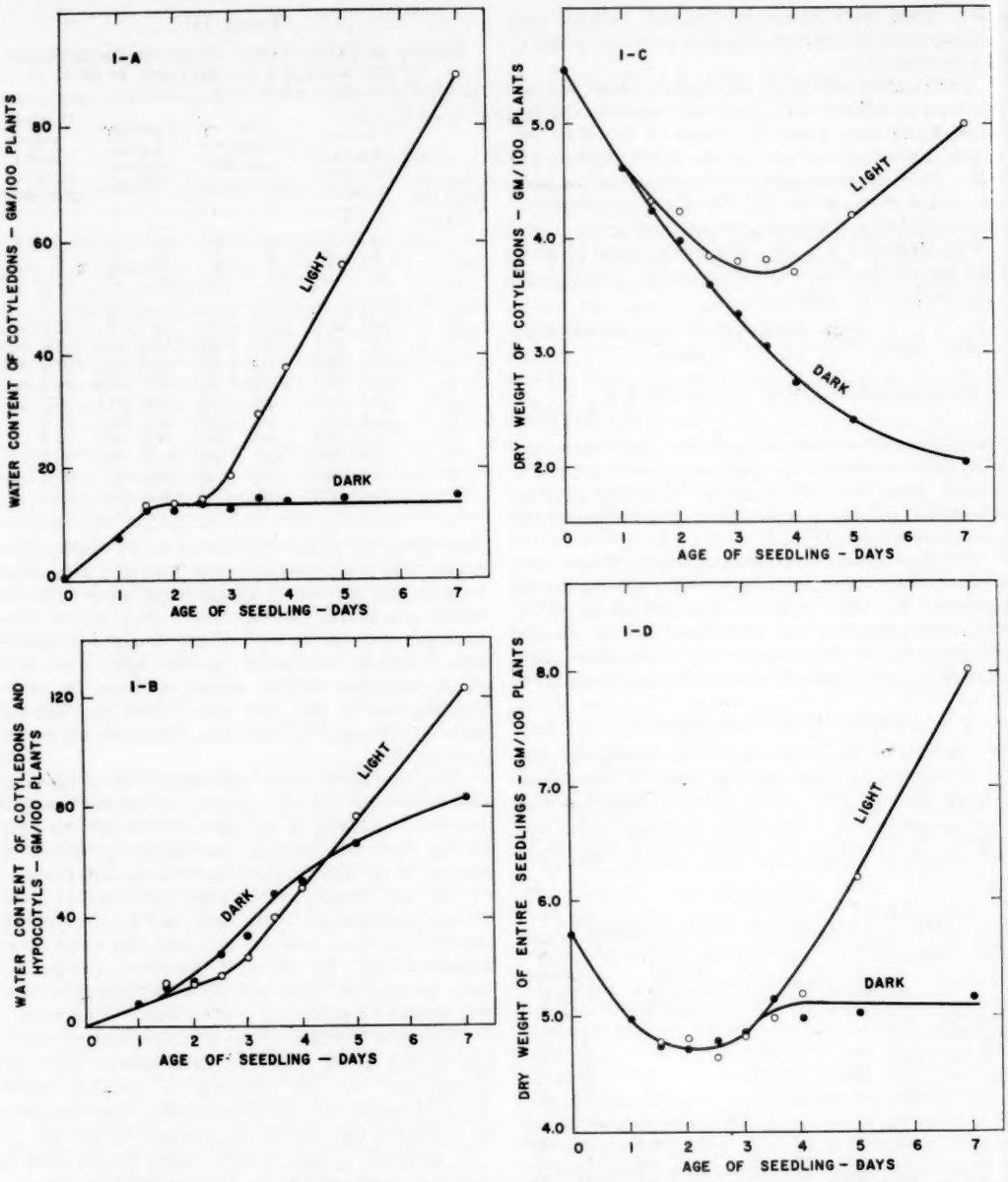

FIG. 1. Growth of cotton seedlings at 30° C in dark and in light: (A) water content of cotyledons; (B) water content of hypocotyls and cotyledons; (C) dry weight of cotyledons; (D) dry weight of entire seedlings.

grew slowly between layers of moist filter paper, and another group grew more rapidly over culture solution. In the 1st and 2nd groups, respectively, after three days in the dark at 30° C, the fat content was 0.825 g and 0.640 g per 200 cotyledons, the combined dry weight of 100 hypocotyls and roots was 1.288 g and 1.632 g, and the free fatty acid content was 0.018 g and 0.015 g per 200 cotyledons.

Another experiment showed that free fatty acids increased in the harvested cotyledons if enzyme activity was not quickly arrested. Cotyledons from two-day-old plants grown over culture solution were dried by being put at once into a vacuum oven at 50° C. They had an abnormally high free fatty acid content, 135 mg per 200 cotyledons, as compared with only 17 mg (table I) in cotyledons in which

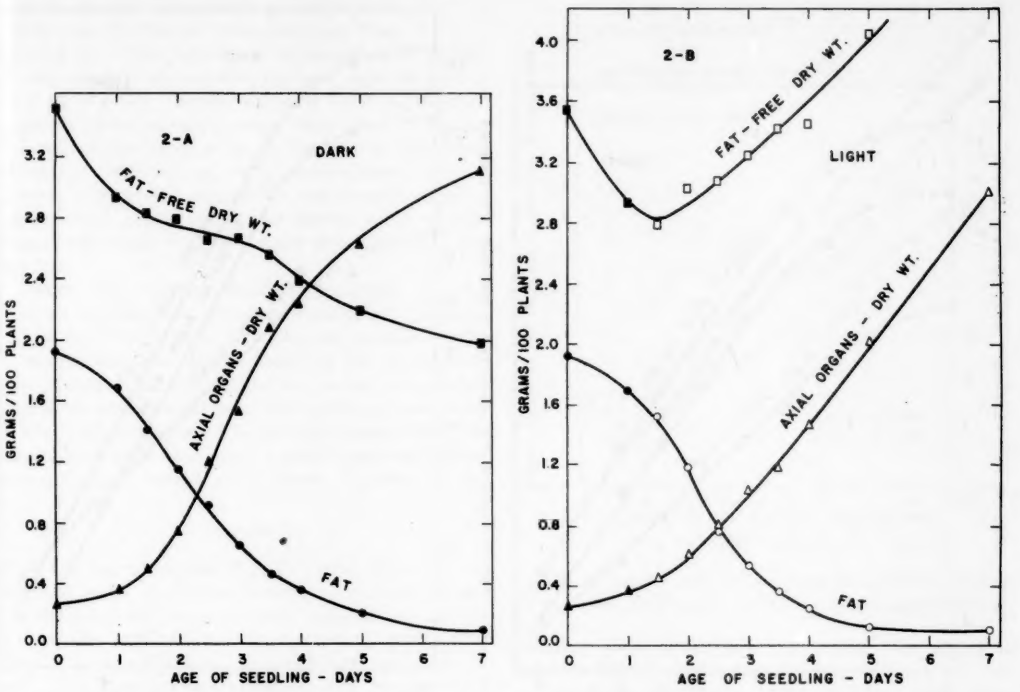

FIG. 2. Changes in fat content and fat-free dry weight of cotyledons, and growth of axial organs (hypocotyls and roots) in dark (A) and in light (B).

enzyme activity had been destroyed by treatment with boiling water.

THE FAT RESERVE: Figure 2 shows the changes in fat content and the fat-free dry weight of the cotyledons with the increase of dry matter in the remainder of the seedling.

The rate of fat loss in the illuminated and in the unilluminated seedlings increased, reached a maximum, and then declined. The sigmoid curve thus formed, however, did not follow closely the equation for an autocatalytic monomolecular reaction. The steep slope of the curves from one through four days showed that fat utilization in the developing seedlings was extremely rapid.

After two days the fat content of the cotyledons was lower in the light than in the dark (table II). But this was not dependent upon the particular light conditions imposed in these experiments. Other tests were made in which seedlings were allowed to develop (1) in continuous light of too low an intensity (2100 meter candles) to give a gain in dry weight even after 7 days, and (2) in alternating 12-hour periods of light (5300 meter candles) and dark. In both cases the fat content of the cotyledons at the end of three and four days was essentially the same as that recorded in table II for the plants of the same age in continuous light (5300 meter candles).

A plot of the logarithm of the fat content against time, after a growth period of two days, gives a straight line (fig 3). Therefore the loss followed the equation for a first-order chemical reaction. That is,

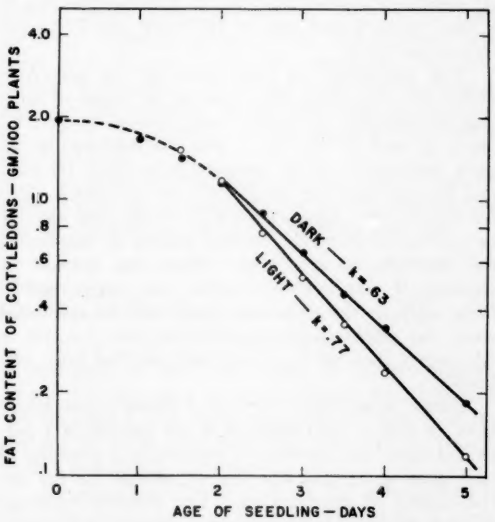

FIG. 3. Fat content of cotyledons in dark and in light. (Logarithmic vertical scale.)

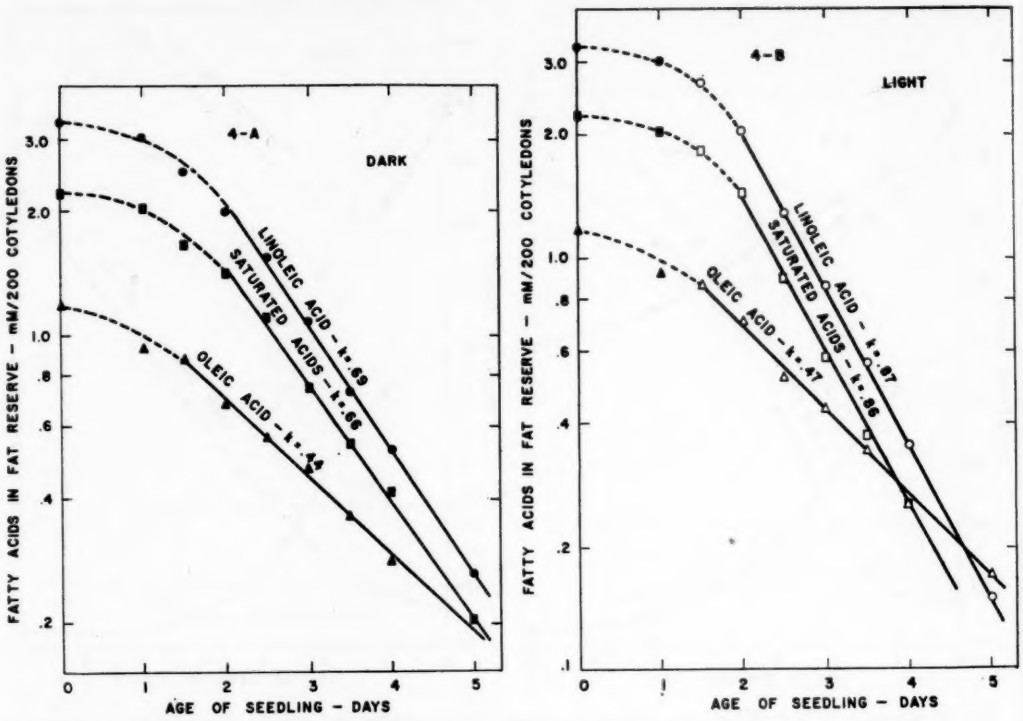

FIG. 4. Depletion of fatty acids in fat reserve during growth of cotton seedlings at 30° C in dark (A) and in light (B). (Vertical scale is logarithmic.)

the rate of loss was constantly proportional to the fat content of the cotyledons. The calculated velocity constants showed that the fat was disappearing at a rate of 63 % per day in the dark and 77 % per day in the light.

The proportion of fatty acids in the glycerides (table II) was above 90 % during all stages of seedling development in the dark and during the first 2.5 days, at least, in the light. For the seedlings in the dark, subtraction of the unsaponifiable from the neutral matter gave the weight of the glycerides. But for those in the light, chlorophyll would also have to be subtracted from the neutral matter in calculating the quantity of glycerides. Since the amount of chlorophyll was not determined, the percentage of fatty acids in the glycerides could only be estimated when the weight of the pigment was low—i.e., up to 2.5 days (when 60 % of the seed fat had been utilized).

FATTY ACID DEPLETION AS A FIRST-ORDER REACTION: A plot of the logarithm of the amount of a particular fatty acid in the fat reserve as a function of time gives a straight line (fig 4), except for the initial stages of germination. The velocity constant (k) for the rate of disappearance of each of the fatty acids is shown by the labels in figure 4. This constant for a particular fatty acid was greater for the plants growing in the light than for those growing in the dark. Under either of the growth conditions the constant for oleic acid was considerably smaller than that for linoleic acid or that for the saturated acids.

FATTY ACID COMPOSITION AND IODINE VALUE: During development of the seedlings in the dark and in the light the iodine value decreased (table III). From the seed through 7-days' growth in the dark there was a regularity in the decrease in the percentage of linoleic acid (50.5 to 29.2 %) and in the

TABLE III

IODINE VALUE OF THE FAT RESERVE IN COTTON SEEDLINGS GROWN IN THE DARK AND IN THE LIGHT AT 30° C

AGE OF SEEDLING DAYS	IODINE VALUE	
	DARK	LIGHT
0	108.3	108.3
1	108.9	108.9
1.5	108.2	108.1
2	106.4	106.2
2.5	106.2	106.7
3	105.6	106.0
3.5	103.6	105.6
4	102.0	103.6
5	100.4	103.9
7	93.3	....

increase in the percentage of oleic acid (18.2 to 45.0 %), as the iodine value decreased from 108.3 to 93.3 (fig 5). The percentage of saturated acids varied only slightly during the 1st four days of growth (31.3 to 31.5 %), thereby showing little dependence on the decrease in iodine value from 108.3 to 102.0.

If all of the fatty acids in the reserve had been constantly depleted at rates proportional to the amounts present in the seed fat, the percentage composition, and consequently the iodine value, during germination would have remained the same as in the seed fat.

Fatty Acid Composition in Relation to the Amount of Reserve Fat: A logarithmic graph is used in figure 6 to show how the composition of the fat reserve changes with depletion of the fat reserve. It is evident that the depletion of the component fatty acids was the same in the unilluminated as in the illuminated plants. The double logarithmic plotting gives straight lines over a wide range and shows that each acid varied as a power function of the total. Hence,

$$f = CT^n$$

where f is the millimoles of linoleic acid, oleic acid, or saturated acids in the fat reserve of 200 cotyledons, T is the total millimoles of fatty acids in the fat reserve of 200 cotyledons, C is a constant (value of f when T is 1 millimole), and n is the slope of the line. The constant n represents the change of f with respect to the depletion of T.

Temperature and Fat Content and Composition: It seemed desirable to find out whether variation in the temperature at which the seedlings developed would alter the relations that were shown to exist at 30° C. Seeds were germinated for one day at 30° C in the dark. The seedlings were then placed over culture solution and allowed to develop in the dark at temperatures ranging from 21° to 39° C. The plants were harvested after the hypocotyl had elongated 60 mm.

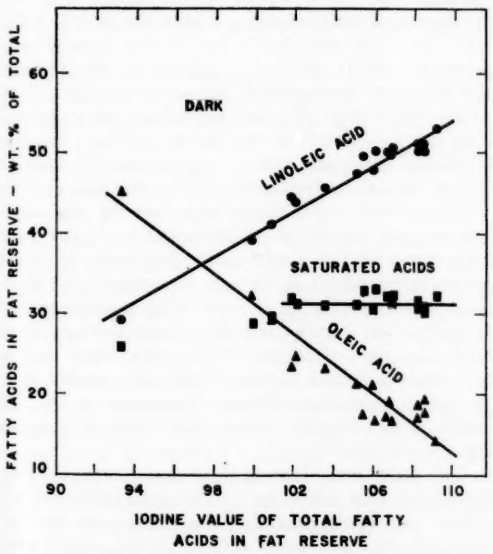

Fig. 5. Variation of fat composition of cotyledons during growth of cotton seedlings at 30° C in dark, in relation to iodine value.

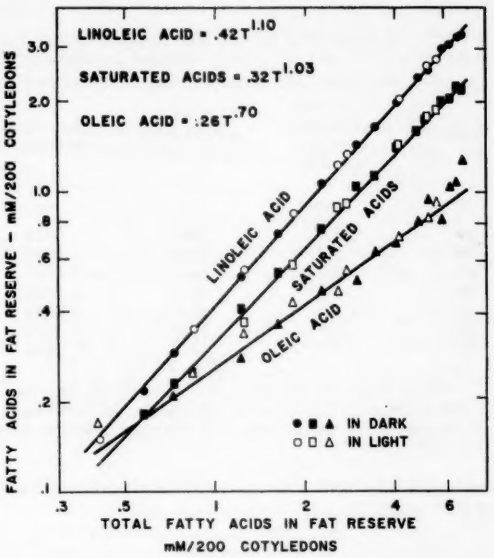

Fig. 6. Fatty acid composition as a power function of the total fatty acids. (Both scales are logarithmic.)

The amount of fat present in the cotyledons was progressively greater as the temperature was raised (table IV). As would be expected, the plants developed much faster, and required less time for their hypocotyl to reach 60 mm, at the higher than at the lower temperatures. The dry weight of the axial organs (hypocotyl and roots) was quite uniform, showing that at each of the temperatures an equal amount of dry matter had been transported out of the cotyledons.

A measure of the efficiency with which the fat reserve is made available to the developing seedling can be obtained by use of the following quotient:

$$\text{Efficiency of fat utilization} = \frac{\text{Increase in dry weight of hypocotyls and roots}}{\text{Loss of fatty acids from the fat reserve}}$$

Substitution of data from table IV in the equation shows that the gain in the combined dry weight of the hypocotyl and roots was 0.95 mg and 1.25 mg per mg of fatty acids removed from the fat reserve at 21° and 36° C, respectively.

A comparison of the data on the composition of the fat reserves in table IV with the graphs in figure 6 shows that the relations found at 30° C between the amount of a fatty acid and the aggregate fatty

TABLE IV

COMPOSITION OF THE FAT RESERVE OF 200 COTYLEDONS AFTER ELONGATION OF THE HYPOCOTYL TO 60 MM AT VARIOUS TEMPERATURES IN THE DARK

TEMP.	TIME * FOR HYPOCOTYL TO REACH 60 MM	DRY WT OF HYPOCOTYLS AND ROOTS	DRY WT OF COTYLEDONS	TOTAL FATTY ACIDS IN FAT RESERVE		COMPOSITION OF FAT RESERVE		
						LINOLEIC ACID	OLEIC ACID	SATURATED ACIDS
°C	days	mg	mg	mg	mM	mM	mM	mM
21	4.6	1366	3375	553	2.02	.95	.41	.66
24	2.8	1404	3479	671	2.46	1.17	.47	.82
27	2.0	1443	3367	663	2.44	1.14	.47	.83
30	1.7	1338	3482	729	2.67	1.28	.52	.87
33	1.5	1596	3413	730	2.67	1.28	.47	.92
36	1.3	1328	3570	840	3.08	1.50	.50	1.08
39	1.7	1315	3546	894	3.27	1.59	.60	1.08

* Time that the seedlings developed at the different temperatures. All seeds were germinated at 30° C for 1 day, at which time, on the basis of 100 plants, the total fatty acids in the fat reserve of the cotyledons was 1616 mg, the dry weight of the cotyledons was 4605 mg, and the combined dry weight of the hypocotyl and roots was 360 mg.

acids were also true throughout the temperature range of 21° to 39° C.

DISCUSSION

In germinating seeds of cotton a large fraction of the intermediate products of fatty acid metabolism is converted into carbohydrate rather than oxidized to CO_2. For example, since the rapidly growing axial organs of a seedling contain only a small amount of fat (2, 11), figure 2 shows that such conversion must have been chiefly responsible for the gain in dry weight of the hypocotyls and roots.

Although carbohydrate produced by photosynthesis might perhaps be expected to retard the depletion of the fat reserve, light was found to accelerate seedling development and to increase the rate of fat utilization (table II). But there was not much opportunity for current photosynthate to exert a sparing effect on fat utilization. Four days were required before the dry weight of the seedlings in the light became greater than that of the seedlings in the dark (fig 1 D). By this time 87 % of the seed fat had disappeared (table II).

A low content of free fatty acids, similar in amount to that in the cotyledons of cotton seedlings during all stages of fat utilization (table I), has been reported for other germinating seeds (2, 11).

Fat disappearance from the cotton seedlings followed the equation for a first-order reaction from the 2nd through the 5th day (fig 3), and the rate was therefore constantly proportional to the fat content. From the seed through the 2nd day, however, the loss was less rapid than after two days. During this initial stage it is possible that the rate was limited by low activity of an enzyme, or of several enzymes, required for fat hydrolysis or fatty acid oxidation.

If it is assumed that before fat oxidation takes place, the fatty acids are split by hydrolysis from the glycerol molecule, then fat breakdown in the early stage of germination could have been retarded by a build-up of hydrolysis products or limited by low lipase activity. There was little accumulation of free fatty acids, or presumably of glycerol, during any stage of germination (table I). Evidence, however, for an initially low lipase activity appears in the literature. Karon and Altschul (8) suggested that the autocatalytic reaction which they found typical of free fatty acid formation in moist cotton seeds might be dependent on an increase in lipase activity with time. Olcott and Fontaine (14) could not detect lipase in dormant cotton seeds, but in another study they (15) showed that during germination the activity of this enzyme increased to a maximum.

A comparison of some of the data of Olcott and Fontaine (15) with results of this study indicates that lipase activity may have been the limiting factor in fat utilization during the 1st two days of germination. When the water content of the seedlings had increased to essentially the same amount (78 % in the Olcott and Fontaine paper, and 80 % in this study), the activity of the fat-hydrolyzing enzyme reached a maximum (Olcott and Fontaine) and fat removal became a first-order reaction (present study).

Straight-line correlations between fatty acid composition and iodine value, illustrated in figure 5 for germinating cotton seeds, have also been found in the mature seed fat of cotton. Stansbury and Hoffpauir (19) showed an inverse relation between the proportion of linoleic and oleic acid as the iodine values changed. Grindley (4) reported that linoleic acid, oleic acid, and the saturated acids increased in the same proportion during deposition of the fat reserve in cotton seeds; consequently the iodine value remained steady.

The slope of the lines in figure 6 shows that during germination the depletion of the saturated acids ($n = 1.03$) was approximately proportional to the decrease in the total fatty acids. Linoleic acid, however, disappeared relatively more rapidly ($n = 1.10$), and oleic acid relatively more slowly ($n = 0.70$), than the total fatty acids. These relations indicate either

that (1) linoleic acid was preferentially removed from the fat reserve, the saturated acids were continually withdrawn in amounts proportional to their mole fractions in the seed fat, and oleic acid was preferentially retained in the fat reserve, or that (2) a conversion of linoleic to oleic acid was taking place on the fat molecule while each of the types of fatty acids was being removed at a rate proportional to its mole fraction.

It would be more difficult to explain a mechanism for a preferential removal of the fatty acids than for a conversion in the fat reserve of some of the linoleic acid to oleic acid. Moreover, evidence from work on the deposition of fat in the maturing seed seems to favor this alternative. Simmons and Quackenbush (17) found that when labeled sucrose was supplied to cuttings of developing soybeans, radioactivity appeared first in oleic acid. They also gave evidence which strongly indicated the conversion of oleic acid to linoleic acid, and of the latter to linolenic acid. If the processes in germination are the reverse of those occurring in seed maturation, the second alternative given above may be the correct one.

Summary

1. Cotton seedlings were grown in solution culture at 30° C in continuous light and in complete darkness. The plants were harvested at various times during the 7-day growth period. Measurements of the development of the cotyledons, hypocotyls, and roots were made. The rate of utilization and the composition of the fat reserve of the cotyledons was determined.

2. After a large loss in the 1st 1.5 days, the dry weight of the seedlings in the dark did not continue to decrease.

3. Current photosynthate exerted no apparent sparing action on depletion of the fat reserve. Fat utilization was more rapid in the light than in the dark.

4. During all stages of fat utilization there was only a small and relatively constant amount of free fatty acids in the cotyledons.

5. From two through five days the rate of fat disappearance followed the equation for a first-order chemical reaction. In like manner the rate of depletion of linoleic acid, oleic acid, and the saturated acids was constantly proportional to the amount of each of these acids, respectively, in the fat reserve.

6. During utilization of the storage fat, the iodine value and the percentage of linoleic acid decreased, the percentage of oleic acid increased, and the percentage of saturated acids remained steady during the 1st four days and then dropped.

7. When seedlings were allowed to develop in the dark in temperatures ranging from 21° to 39° C, there was no alteration in the pattern of fat utilization found at 30° C.

8. Under all germination conditions tested, the fat composition was the same function of the total amount of fatty acids remaining in the fat reserve; it was independent of the rate or type of seedling development. Linoleic acid and the saturated acids were depleted relatively faster than oleic acid.

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Literature Cited

1. American Oil Chemists' Society, Chicago. Official and tentative methods of analysis. 1946, rev. to 1952.
2. Crombie, W. M. and Comber, R. Fat metabolism in germinating *Citrullus vulgaris*. Jour. Exptl. Bot. 7: 166–180. 1956.
3. Daggs, R. G. and Halcro-Wardlaw, H. S. The conversion of fat to carbohydrate in the germinating castor bean. II. The combustion respiratory quotient as determined by a modified oxycalorimeter. Jour. Gen. Physiol. 17: 303–309. 1933.
4. Grindley, D. N. Changes in composition of cotton seeds during development and ripening. Jour. Soc. Food Agric. 1: 147–151. 1950.
5. Hilditch, T. P. and Maddison, L. The mixed unsaturated glycerides of liquid seed fats. II. Low-temperature crystallisation of cottonseed oil. Jour. Soc. Chem. Ind. (London) 59: 162–168T. 1940.
6. Holman, R. T. Lipoxidase activity and fat composition of germinating soybeans. Arch. Biochem. 17: 459–466. 1948.
7. Johnston, F. A. and Sell, H. M. Changes in chemical composition of tung kernels during germination. Plant Physiol. 19: 694–698. 1944.
8. Karon, M. L. and Altschul, A. M. Effect of moisture and of treatments with acid and alkali on rate of formation of free fatty acids in stored cottonseed. Plant Physiol. 19: 310–325. 1944.
9. MacLachlan, P. L. Fat metabolism in plants with special reference to sterols. II. Differential changes in the cotyledons and in the roots, stems and leaves. Jour. Biol. Chem. 114: 185–191. 1936.
10. Matthes, E. Physiologische Untersuchungen über Umwandlungen des Oeles in keimenden Sonnenblumensamen. Bot. Arch. 19: 79–135. 1927.
11. Miller, E. C. A physiological study of the germination of *Helianthus annuus*. Annals Bot. 24: 693–726. 1910.
12. Miller, E. C. A physiological study of the germination of *Helianthus annuus*. II. The oily reserve. Annals Bot. 26: 889–901. 1912.
13. Murlin, J. R. The conversion of fat to carbohydrate in the germinating castor bean. I. The respiratory metabolism. Jour. Gen. Physiol. 17: 283–302. 1933.
14. Olcott, H. S. and Fontaine, T. D. The absence of lipase in cottonseed. Oil and Soap 18: 123–124. 1941.
15. Olcott, H. S. and Fontaine, T. D. Composition of cottonseeds. IV. Lipase of germinated seed. Jour. Amer. Chem. Soc. 63: 825–827. 1941.
16. Pierce, H. B., Sheldon, D. E. and Murlin, J. R. The conversion of fat to carbohydrate in the germinating castor bean. III. The chemical analysis and correlation with respiratory exchange. Jour. Gen. Physiol. 17: 311–325. 1933.

17. SIMMONS, R. O. and QUACKENBUSH, F. W. The sequence of formation of fatty acids in developing soybean seeds. Jour. Amer. Oil Chemists' Soc. 31: 441–443. 1954.
18. STANSBURY, M. F. and GUTHRIE, J. D. Storage of cottonseed and peanuts under conditions which minimize changes in chemical composition. Jour. Agr. Res. 75: 49–62. 1947.
19. STANSBURY, M. F. and HOFFPAUIR, C. L. Relation between fatty acid composition and iodine value of cottonseed oil. Jour. Amer. Oil Chemists' Soc. 29: 53–55. 1952.
20. TRELEASE, S. F. Chamber with thermostatic control and rotating table for plant cultures. Bull. Torrey Bot. Club 52: 389–396. 1925.

PHYSIOLOGICAL STUDIES ON ACID METABOLISM IN GREEN PLANTS. VI. TRANSAMINASES IN CELL-FREE EXTRACTS FROM KALANCHOË LEAVES[1,2]

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Leaves of Kalanchoë and Bryophyllum and of other plants which exhibit the essential features of Crassulacean acid metabolism are able to fix, in the dark, considerable amounts of CO_2 into carboxylic acids (11, 12, 13) and the acid which accumulates is normally very largely malic (20, 7, 6). When the products of fixation over the course of a few hours are studied by the use of $C^{14}O_2$, varying but usually small proportions of the C^{14} incorporated appear in compounds other than malate. Among these are aspartic and glutamic acids, and alanine (14, 15, 13, 10).

The discovery of phosphoenolpyruvic carboxylase (1) and, more recently, its demonstration in leaves of Kalanchoë and other Crassulacean plants (9, 16) have provided new information about the actual mechanism of dark CO_2 fixation, and it now seems reasonably certain (9, 17, 18) that the synthesis of oxalacetate by this enzyme must be an important step in the dark CO_2 fixation and the resulting acidification in such leaves. Malate would then be produced by the rapid reduction of oxalacetate (OAA) by the active malic dehydrogenase present (17).

In an examination of the products of a 6-second exposure of young Bryophyllum leaves to $C^{14}O_2$ in the dark, Saltman et al (9) found that malic acid contained most of the isotope. The only other product detected was aspartate. From this and other evidence they concluded that the first product of the CO_2 fixation was in fact oxalacetate, and that the malate and aspartate were secondary products arising from reduction and transamination, respectively, of the OAA. Labelled alanine and glutamate appearing among the products of longer periods of $C^{14}O_2$ fixation were attributed to transaminations involving labelled pyruvate and α-oxoglutarate derived from the OAA (10).

The present paper reports the occurrence of glutamic-aspartic and glutamic-alanine transaminases in cell-free extracts of mature leaves of *Kalanchoë crenata*, and that the former enzyme can be linked with phosphoenolpyruvic carboxylase in the extracts to yield labelled aspartate from phosphoenolpyruvate and $NaHC^{14}O_3$. Previous work (3) suggests that, in the intact mature leaves, the phosphoenolpyruvate which is carboxylated to yield oxalacetate is itself a product of the dark CO_2 fixation. These findings are discussed in relation to the appearance of labelled amino acids among the early products of dark $C^{14}O_2$ fixation in the intact leaves.

MATERIALS AND METHODS

Leaf extracts were prepared from fully matured leaves of *Kalanchoë crenata* by a method which has been described previously (17). To the supernatant solution resulting from the first high speed centrifugation, sufficient ammonium sulphate was added to make a 60 % (w/v) solution of the added salt. The mixture was allowed to stand overnight at – 4° C. The resulting protein precipitate was then centrifuged and resuspended in saturated ammonium sulphate for storage at – 30° C. When required, the protein was again separated and dissolved in a minimum volume of demineralized water. The resulting solution, which contained several enzymes including phosphoenolpyruvate carboxylase, malic dehydrogenase, and transaminases, was dialyzed against demineralized water for four hours immediately before use. Extracts prepared in this way showed high transaminase activity without the addition of pyridoxal phosphate.

Spectrophotometric measurements of the oxidation of DPNH at 340 mμ were made at 25° C with a Unicam S.P. 500 in cells (d = 1 cm) containing a total of 3 ml fluid. Reaction mixtures are detailed below either in the text or in the legends to figures.

For chromatographic analysis, reactions were stopped after the required time intervals by the addition of formic acid. The mixtures were then concentrated and appropriate aliquots chromatographed in one or two dimensions on Whatman No. 1 filter paper with tertiary amyl alcohol/formic acid/water (3/1/3) and *n*-propanol/ammonia/water (6/3/1) as developing solvents (see, e.g., 8). Radioautographs were prepared by exposing sheets of Ilford "Ilfex"

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[2] This study was supported in part by a Grant-in-aid from the Royal Society to one of us (S.L.R.).
[3] Imperial Chemical Industries Research Fellow.

film to the chromatograms for 7 days. Carboxylic acids on the chromatograms were detected by spraying with aqueous bromocresol purple solution, and amino acids by their reaction with ninhydrin.

In the chromatography of extracts containing compounds labelled with C^{14}, the extract was applied to the chromatogram together with sufficient of a mixture of marker acids to allow positive location of the marker compounds for comparison with active areas defined on the radioautographs.

Experimental Results

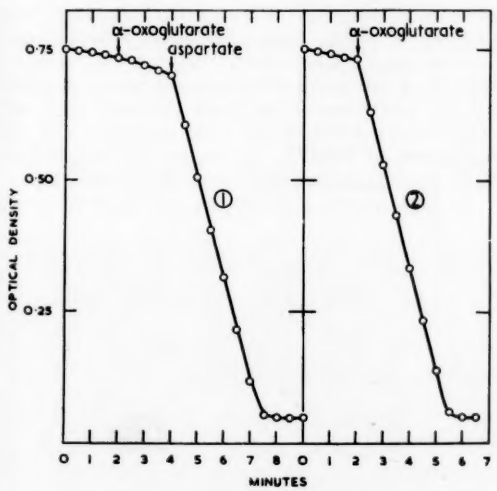
Aspartic-Glutamic Transaminase: The presence in the leaf extract of an enzyme system catalyzing the transfer of amino groups from aspartic acid to α-oxoglutaric acid (equation i) was demonstrated by linking the system with the malic dehydrogenase also present in the extracts (equation ii) and measuring the oxidation of reduced DPN spectrophotometrically in the combined reaction (equation iii).

α-oxoglutarate + aspartate ⟶ glutamate + oxalacetate (i)

oxalacetate + DPNH ⟶ malate + DPN (ii)

α-oxoglutarate + aspartate + DPNH ⟶ glutamate + malate + DPN (iii)

The oxidation of DPNH as indicated by the decrease in optical density (see fig 1) was very slow in reaction mixtures to which neither α-oxoglutarate nor aspartate had been added, and there was no appreciable increase in the rate of oxidation on the subsequent addition of α-oxoglutarate. However, when the system was completed by the addition of aspartate, a rapid linear fall in optical density occurred. In a similar experiment (fig 2) when aspartate was added first to a reaction mixture containing enzyme and DPNH, the oxidation was again very slow, but the addition of α-oxoglutarate after two minutes, to complete the system, resulted in a rapid oxidation of the DPNH.



Figs. 1 and 2. Oxidation of DPNH in equation iii. Each spectrophotometer cell initially contained 0.1 ml Kalanchoë extract, 0.1 ml of 10% 2-amino-2 hydroxymethylpropane-1:33-diol (TRIS) at pH 7.4 and 2.6 ml of mineral free water. Additions (fig 1): At zero time 0.1 ml of DPNH (approx. 0.35 micromole); at 2 minutes 0.1 ml of α-oxoglutarate (10 micromoles); at 4 minutes 0.1 ml of aspartate (10 micromoles). Additions (fig. 2): At zero time, 0.1 ml of DPNH (approx. 0.35 micromole) and 0.1 aspartate (10 micromoles); at 2 minutes 0.1 ml of α-oxoglutarate (10 micromoles).

A complete mixture containing leaf extract, 0.35 micromoles DPNH, 10 micromoles of aspartate, 10 micromoles of α-oxoglutarate, all at pH 7.4, was incubated at 30° C for 25 minutes. Chromatography confirmed that glutamate was produced at the expense of aspartate.

As shown previously (17) Kalanchoë leaf extracts contain phosphoenolpyruvic carboxylase, and when supplied with PEP, $NaHC^{14}O_3$, and $MgCl_2$, synthesize oxalacetate, incorporating C^{14} in the β-carboxyl group (equation iv). With the further addition of DPNH to the reaction mixture the oxalacetate is reduced to malate by malic dehydrogenase present in the extract (equation v). The result of such an experiment

$$PEP + CO_2 \longrightarrow \text{oxalacetate} + \text{orthophosphate} \quad \text{(iv)}$$

$$PEP + CO_2 + DPNH \longrightarrow \text{malate} + \text{orthophosphate} + DPN \quad \text{(v)}$$

performed with the present extracts is illustrated in figure 3 a, which shows a radioautograph prepared from a two-dimensional chromatogram of the products of the reaction. The main active area of the chromatogram corresponded exactly with the location of malic acid. The trace of radioactive fumarate (F in fig 3 a) also present can be attributed to the presence of fumarase in the crude leaf extract.

In the above experiment phosphoenolpyruvic carboxylase was linked with malic dehydrogenase and the end product was malate. In another experiment glutamate was substituted for the DPNH in the reaction mixture (see legend to fig 3 b), and after a period of incubation, the products were chromatographed and a radioautograph prepared from the developed chromatogram. The result shown (fig 3 b) demonstrated the production of aspartate containing C^{14}. The synthesis of this labelled acid is attributed to the transfer of amino groups from glutamate to the labelled oxalacetate resulting from the CO_2 fixation catalyzed by phosphoenolpyruvic carboxylase. The overall reaction can be represented by equation vi.

$$PEP + C^{14}O_2 + \text{glutamate} \longrightarrow \text{aspartate (C-14)} + \alpha\text{-oxoglutarate} + \text{orthophosphate} \quad \text{(vi)}$$

When a third reaction mixture containing plant extract, PEP, $NaHC^{14}O_3$, glutamate, DPNH, and $MgCl_2$, was incubated at 25° C for 20 minutes and then analyzed by chromatography and radioautogra-

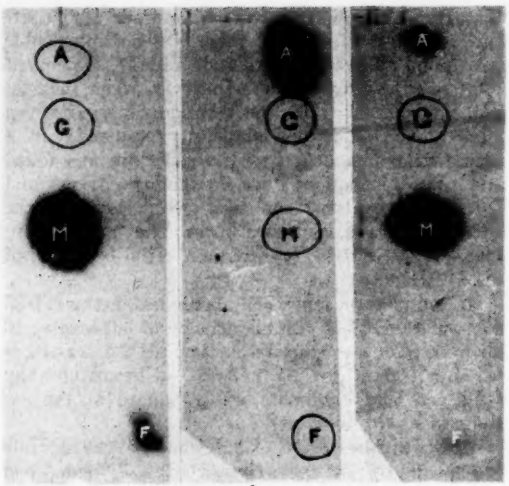


FIG. 3. Radioautographs of chromatograms showing incorporation of $C^{14}O_2$ (a) into malate (equation v) (b) into aspartate (equation vi) and (c) into malate and asparate (equation v and vi). Reaction mixtures: (a) Kalanchoë extract, $NaHC^{14}O_3$ (25 μC, 25 micromoles), PEP (10 micromoles), DPNH (approx. 3.5 micromoles), $MgCl_2$ (3 micromoles); all at pH 7.4. (b) As for (a) but with glutamate (10 micromoles) in place of DPNH. (c) As for (a) but with glutamate (10 micromoles) added. Key; M = malate, A = aspartate, G = glutamate, F = fumarate. The dark areas indicate the location of compounds containing C^{14}.

phy the radioactive products were malate and aspartate (fig 3 c). The C^{14} content of the malate was about 10 times that of the aspartate. The simplest explanation of this result is that the OAA synthesized by the carboxylase served as a substrate for both the dehydrogenase and the transaminase in the plant extract. In fact the reaction mixture used in the above experiments appears to constitute a cell-free model of young Bryophyllum leaves (9) to the extent that the same products, namely malate and aspartate, are labelled in a short period of $C^{14}O_2$ fixation. Moreover, the distribution of isotope between the compounds is similar.

GLUTAMIC-ALANINE TRANSAMINASE: Evidence of the presence in the leaf extract of an enzyme transferring, directly or indirectly, amino groups from glutamate to pyruvate to form alanine was provided in an experiment in which the leaf extract was incubated with pyruvic acid (3-C^{14}) and glutamate for 30 minutes at 25° C. The reaction mixture was then chromatographed in two dimensions with added carrier amino acids including aspartic, glutamic, and alanine. A radioautograph of the developed chromatogram showed only one active area which corresponded exactly with the location of alanine (see fig 4).

DISCUSSION

The results reported above indicate that crude extracts from mature Kalanchoë leaves contain active glutamic-aspartic and glutamic-alanine transaminases in addition to the phosphoenolpyruvic carboxylase, malic dehydrogenase and other enzymes known to be present from previous work (17). Further, it has been demonstrated that the glutamic-aspartic transaminase can be linked with the carboxylase to yield labelled aspartate from PEP and $C^{14}O_2$ in the presence of glutamate. When DPNH was also added to the reaction mixture, the labelled products were malate and aspartate.

The transaminases detected would, in the presence of glutamate, yield from OAA and pyruvate, respectively, aspartate and alanine. Moreover, they would account for the production of glutamate from α-oxoglutarate, in the presence of aspartic acid or alanine. Since the three amino acids mentioned are those which are present in the highest concentrations in the free state in Kalanchoë leaves, the presence of their C^{14} labelled analogues among the early products of dark $C^{14}O_2$ fixation can readily be explained if oxalacetate, pyruvate and α-oxoglutarate rapidly incorporate the isotope. The presence of these keto acids among the labelled products after longer periods of $C^{14}O_2$ fixation by mature Kalanchoë leaves has been demonstrated (2), and it seems highly probable that they must be produced very early in the sequence of reactions following CO_2 fixation. The evidence is strong that phosphoenolpyruvate carboxylase plays a major part in the dark CO_2 fixation; hence OAA production would precede malate accumulation. Amination of the OAA produced in a leaf supplied with $C^{14}O_2$ would yield aspartate, and the appearance of label in the amino acid might well occur almost simultaneously with that in malate.

If, however, the only CO_2 fixation involved in the

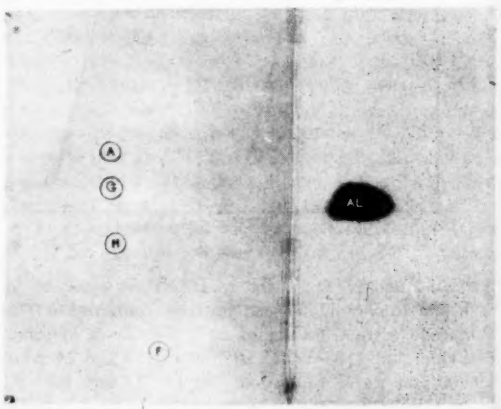
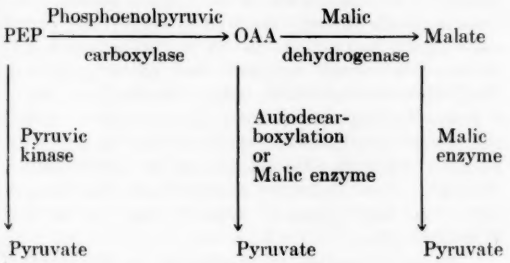


FIG. 4. Production of labelled alanine by transamination. Reaction mixture contained Kalanchoë extract, glutamate (15 micromoles) and $C^{14}H_3COCOONa$ (15 μC, 15 micromoles); all at pH 7.4. Key; G = glutamate, A = aspartate, M = malate, F = fumarate, AL = alanine.

synthesis of malate, in vivo, were that catalyzed by phosphoenolpyruvic carboxylase, the OAA and malate synthesized would contain the carbon isotope initially only in the β-carboxyl group. Hence any pyruvate derived from these acids would presumably be unlabelled. To account for the rapid production of labelled pyruvate in young Bryophyllum leaves it has, however, been suggested (10) that if OAA is converted to malate, and the malate is in equilibrium with fumarate under the influence of fumarase, the malate and oxalacetate would eventually acquire label in both carboxylic groups; consequently pyruvate produced by decarboxylation would also be labelled.

For mature Kalanchoë leaves, the rapid production of labelled pyruvate can be explained more directly. Bradbeer et al (3) have noted that in these leaves the malate synthesized from $C^{14}O_2$ always contains only about two thirds of the isotope in the β-carboxyl, the remaining one third being in the α-carboxyl group, whatever the period of exposure to the gas. As a result of this apparently constant distribution, they have suggested that the PEP precursor of malate is itself labelled before the further incorporation of $C^{14}O_2$ to yield oxalacetate. The PEP, so formed, could then give rise to labelled pyruvate by the action of one or more of the enzymes, pyruvic kinase, phosphoenolpyruvic carboxylase, malic enzyme, malic dehydrogenase, as indicated in the scheme outlined below. With the exception of the



kinase all of these enzymes have been shown to be present in mature leaves of Kalanchoë (4, 17, 19). From the resulting pyruvate, C^{14}-labelled alanine would appear in the presence of glutamic acid-alanine transaminase.

The consumption of oxalacetate labelled in both carboxylic groups by way of the normal reactions of the Krebs acid cycle would yield α-oxoglutarate containing the C^{14} label of only the β-carboxyl group of the OAA. Either of the freely reversible transaminases detected in Kalanchoë leaves could account for the production of labelled glutamate from such α-oxoglutarate.

The reactions discussed above do not bring about any net synthesis of amino acids. Nor in fact has any appreciable synthesis of amino acids been detected in detached Kalanchoë leaves undergoing rapid dark acidification (5). Nevertheless, in leaves of the intact plant, oxalacetate and keto acids derived from it may play essential roles in amino acid metabolism, and it is not improbable that the carboxylation of PEP and transaminations of the types discussed above might be linked to serve a useful purpose in many plants.

Summary

Aspartic-glutamic transaminase in cell-free extracts of mature Kalanchoë leaves has been demonstrated by spectrophotometric and chromatographic methods. Glutamic-alanine transaminase has been demonstrated chromatographically in the same preparations by the use of radioactive pyruvate.

In the presence of appropriate reaction mixtures containing $NaHC^{14}O_3$, this aspartic-glutamic transaminase has been linked with phosphoenolpyruvic carboxylase and malic dehydrogenase, which are also present in the leaf extracts, so as to produce either labelled aspartate or labelled malate and aspartate.

These results have been discussed in relation to the parts played by transaminases in the production of labelled amino acids when intact mature leaves of Kalanchoë are fed with $C^{14}O_2$ in the dark.

The authors wish to express their thanks to Professor M. Thomas, F.R.S., for his continued interest and assistance in this work.

Literature Cited

1. Bandurski, R. S. and Greiner, C. M. The enzymatic synthesis of oxalacetate from phosphorylenolpyruvate and carbon dioxide. Jour. Biol. Chem. 204: 781–786. 1953.
2. Bradbeer, J. W. Some carbohydrate and acid conversions in *Kalanchoë crenata*. Ph.D. Thesis, Durham University (Newcastle Division, King's College) 1954.
3. Bradbeer, J. W., Ranson, S. L. and Stiller, Mary Malate synthesis in Crassulacean leaves. I. The distribution of C^{14} in malate in leaves exposed to $C^{14}O_2$ in the dark. Plant Physiol. 33: 66–70. 1958.
4. Clendenning, K. A., Waygood, E. R. and Weinberger, P. The carboxylases of leaves and their role in photosynthesis. Canadian Jour. Bot. 30: 395–409. 1952.
5. Lyndon, R. F. Studies on the nitrogen metabolism of Kalanchoë leaves in the dark in relation to acidification and deacidification and gaseous exchange. Ph.D. Thesis, Durham University (Newcastle Division, King's College) 1957.
6. Moyse, A. and Jolchine, G. Les variations quantitatives des acides organiques des feuilles de Bryophyllum à l'obscurite et à la lumiere, en fonction de la tension partielle de l'oxygen. Bull. soc. chim. biol. 38: 761–784. 1956.
7. Pucher, G. W., Leavenworth, G. S., Ginter, W. D. and Vickery, H. B. Studies in the metabolism of Crassulacean plants: The behaviour of excised leaves of *Bryophyllum calycinum* during culture in water. Plant Physiol. 32: 477–493. 1947.
8. Ranson, S. L. Non volatile mono-, di- and tricarboxylic acids (Chromatographic and ion exchange methods). In: Modern Methods of Plant Analysis. K. Paech and M. V. Tracey, eds. Vol. 2. Pp. 539–582. Springer-Verlag, Berlin 1955.
9. Saltman, P., Kunitake, G., Spolter, H. and Stitt,

C. The dark fixation of CO_2 by succulent leaves. Plant Physiol. 31: 464–468. 1956.
10. Saltman, P., Lynch, V. H., Kunitake, G. M., Stitt, C. and Spolter, H. The dark fixation of CO_2 by succulent leaves: Metabolic changes subsequent to the initial fixation. Plant Physiol. 32: 197–200. 1957.
11. Thomas, M. Plant Physiology. Pp. 320–340. J. and A. Churchill, London (3rd Edition) 1947.
12. Thomas, M. and Beevers, H. Physiological studies on acid metabolism in green plants. II. Evidence of CO_2 fixation in Bryophyllum and the study of diurnal fluctuation of acidity in this genus. New Phytologist 48: 421–447. 1949.
13. Thomas, M. and Ranson, S. L. Physiological studies on acid metabolism in green plants. III. Further evidence of CO_2 fixation during dark acidification of plants showing Crassulacean acid metabolism. New Phytologist 53: 1–30. 1954.
14. Thurlow, J. and Bonner, J. Fixation of atmospheric CO_2 in the dark by leaves of Bryophyllum. Arch. Biochem. 19: 500–511. 1948.
15. Varner, J. E. and Burrell, R. C. Use of C^{14} in the study of the acid metabolism of *Bryophyllum calycinum*. Arch. Biochem. 25: 280–287. 1950.
16. Walker, D. A. Malate synthesis in a cell-free extract from a Crassulacean plant. Nature 178: 593–594. 1956.
17. Walker, D. A. Physiological studies on acid metabolism. 4. Phosphoenolpyruvic carboxylase activity in extracts of Crassulacean plants. Biochem. Jour. 67: 73–79. 1957.
18. Walker, D. A. and Brown, J. M. A. Physiological studies on acid metabolism. 5. Effects of carbon dioxide concentration on phosphoenolpyruvic carboxylase activity. Biochem. Jour. 67: 79–83. 1957.
19. Waygood, E. R. and Coles, C. H. Isolation of active enzyme systems from Crassulacean plants. Reported at Bot. Soc. of Amer., A.I.B.S. meeting, Sept. 6–10, Madison, Wisconsin 1953.
20. Wolf, J. Beiträge zur Kenntnis des Säurestoffwechsels sukkulenter Crassulaceen. II. Untersuchungen über Beziehungen zwischen Sedoheptose und Äpfelsäure. Planta 26: 516–522. 1937.

STUDIES ON THE BIOSYNTHESIS OF PECTINIC ACID METHYL ESTERS[1,2,3]

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A previous report from this laboratory (4) showed that the methyl group of methionine can be transferred as a unit to form methyl esters of pectinic acid in radish plants. That study has now been extended, in the present work, to examine other compounds which have contributed to the "one carbon" pool in animal and plant metabolism and may also be introduced into pectinic acid methyl esters in the radish plant. Included in the group of methyl precursors tested were glycine, serine, formate and formaldehyde.

Materials and Methods

Preparation of Radish Plants: The radish plant, *Raphanus sativus*, var. Comet, was used throughout the present work. Seeds were sprouted on damp cheese cloth and grown in constantly aerated Hoagland nutrient solution (1) following the procedures used by Sato and co-workers (4). When the enlarged hypocotyl and upper root, hereafter referred to as the "radish," had developed to about 1.0 to 1.5 cm diameter, which generally took 5 to 7 weeks depending on the time of the year, the plant was considered to be ready for the feeding experiment.

Administration of Labeled Compounds: Glycine-2-C^{14}, DL-serine-3-C^{14}, sodium formate-C^{14} and formaldehyde-C^{14} were fed to separate groups of radish plants in the nutrient solution. These compounds had been shown to be readily absorbed by various plants through the root system. The plants were placed individually in 100 ml wide-mouth cylinders which were wrapped with black paper to shield their contents from light. On the first day of a 9-day feeding period, each plant received 0.0134 millimoles of radioactive compound having 6.09×10^4 counts per minute. At the end of the 9-day period, the plants were harvested and pectinic acid was isolated from each group of radishes using the method of Kertesz (2).

The incorporation of carbon-14 in the methyl ester group was ascertained by demethylation of pectinic acid as described by Sato and co-workers (4). In the demethylation procedure the methyl esters were hydrolyzed, and the resulting methanol then converted to $BaCO_3$. Experiments indicated that no scission of S-methyl, N-methyl or methyl ether groups occurred under the conditions of the demethylation procedure. Although no test for purity of the pectinic acid was carried out, the assumption was made that only methyl esters of pectic substances were hydrolyzed and that possible contaminants, such as protein, yielded no methanol in the procedure.

All radioactivities were measured with either the end-window Geiger-Müller tube or an internal flow-tube. The efficiency of the end-window tube as used was 2 %, and the other 20 %.

Results

The radioactivities of pectinic acid isolated from different groups of plants administered various radio-

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[2] These studies were supported by grants from the Atomic Energy Commission and the National Institutes of Health.
[3] Presented in part at the meeting of the Federated Biological Societies, Chicago, March 1957.

TABLE I

COMPARISON OF VARIOUS COMPOUNDS AS METHOXYL GROUP PRECURSORS FOR PECTINIC ACID

EXP. NO. (NO. OF PLANTS)	COMPOUNDS FED	ACTIVITY OF PECTINIC ACID CPM/100 MG × 10^{-3}	ACTIVITY OF METHYL ESTER CARBON (AS $BaCO_3$) FROM 100 MG PECTINIC ACID CPM × 10^{-3}	% ACTIVITY IN METHYL ESTER GROUP OF PECTINIC ACID
1. (40)	Na-formate-C^{14} *	9.57	7.75	82
2. (40)	Na-formate-C^{14}	13.2	9.40	75
3. (25)	Glycine-2-C^{14} **	4.55	3.73	82
4. (25)	Glycine-2-C^{14}	4.18	3.08	73
5. (25)	Serine-3-C^{14} ***	2.74	1.17	43
6. (23)	Serine-3-C^{14}	2.71	0.75	27
7. (12)	Formaldehyde-C^{14} †	9.07	7.57	83
8. (21)	Formaldehyde-C^{14}	6.16	5.24	85

* Obtained from Technical Associates, Burbank, California.
** Obtained from Tracerlab, Inc., Boston, Massachusetts.
*** Obtained from California Foundation for Biochemical Research.
† Obtained from Isotopes Specialties Co., California, and from Volk Radiochemical Co., Chicago, Illinois.

active nutrients are presented in table I. Since the molecular weight of pectinic acid is not known, its radioactivity is expressed as counts per minute per 100 mg. The radioactivity of pectinic acid methyl ester carbon, counted as $BaCO_3$, is also presented in table I. The radioactivity of $BaCO_3$ is expressed as counts per minute obtained from 100 mg pectinic acid. All counts were corrected for self-absorption of the sample.

A comparison of the extent of incorporation of labelled carbon of the various compounds into pectinic acid reveals that the carbon of both formate and formaldehyde is introduced to a greater extent and the alpha-carbon of glycine and the beta-carbon of serine to a lesser extent. When methionine-methyl-C^{14} was administered to a group of radish plants (4) pectinic acid was about as radioactive as it was following administration of glycine-2-C^{14}. The variation in radioactivity of pectinic acid between duplicate experiments after feeding a single material might have been due to seasonal influences in rate of growth and metabolism.

Except after feeding DL-serine-3-C^{14}, the majority of the radioactivity of the pectinic acid could be recovered upon demethylation of pectinic acid. No attempt was made to determine the position in the pectinic acid molecule of the radioactive carbon not located in the methoxyl groups.

DISCUSSION

The general metabolic pattern of formation of methyl ester groups of pectinic acid in the radish plant appears to be similar to the formation of the N-methyl group of nicotine and of O-methyl group of lignin in tobacco plants. However, certain specific differences are noted. The carbon of formate was incorporated into methyl ester groups to a much greater extent than into N-methyl or O-methyl groups. Furthermore, the β-carbon of serine was more randomized in entering pectinic acid than in entering nicotine or lignin in tobacco plants. These findings may represent a difference between metabolism in radish and tobacco plants or, on the other hand, may represent differences in the biosynthesis of methyl ester groups and N-methyl or O-methyl groups.

Bonner and co-workers (3) recently have suggested that indoleacetic acid exerts its effect on plant growth by increasing the rate of transfer of methyl groups from methionine to form pectin methyl esters. Presumably, the esterfied pectin allows for expansion of the cell wall as water is taken in by the cell. Since the present study shows that pectinic acid may be methylated by a process other than by transmethylation from methionine, it would be of interest to measure the effect of indoleacetic acid on the rate of methylation of pectinic acid by glycine, serine, formate and formaldehyde.

SUMMARY

The labelled carbons of glycine-2-C^{14}, serine-3-C^{14}, formaldehyde-C^{14} and formate-C^{14} were incorporated in pectinic acid in radish plant metabolism. From 70 to 80 % of the radioactivity of pectinic acid was found in the methyl ester carbon after feeding glycine, formaldehyde and formate, whereas, about 30 to 40 % of the radioactivity was in the methyl ester carbon after feeding serine. When these methyl precursors were fed under similar conditions, formate was incorporated into methyl esters to the greatest extent followed in order by formaldehyde, glycine and serine.

LITERATURE CITED

1. HOAGLAND, D. R. and ARNON, D. J. The water-culture method for growing plants without soil. California Agr. Exp. Sta. Circular 347. 1950.
2. KERTESZ, Z. I. The Pectic Substances. Pp. 103–116. Interscience Publishers, New York 1951.
3. ORDIN, L., CLELAND, R. and BONNER, J. Methyl esterification of cell wall constituents under the influence of auxin. Plant Physiol. 32: 216–220. 1957.
4. SATO, C. S., BYERRUM, R. U. and BALL, C. D. The biosynthesis of pectinic acid methyl esters through transmethylation from methionine. Jour. Biol. Chem. 224: 717–723. 1957.

A NEW METHOD OF DETERMINING DROUGHT RESISTANCE[1]

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Present methods for testing resistance of plants to frost and drought require long observations in the field under conditions which are unsatisfactory for such an investigation, due to the great variation from year to year of weather conditions and other factors. Attempts to substitute laboratory tests for field experiments in measuring resistance of plants to freezing started with sugar cane some years ago in the author's laboratory. A laboratory test was developed for measuring the resistance of sugar cane to frost. The method was based on the measurement of the drip that cane segments exuded upon thawing, after having been frozen in the laboratory under standard conditions (5). It was shown that the drip given by various frozen products after thawing was closely related to the ability of these materials to withstand freezing storage changes (2, 3, 4). It was considered probable that frost hardiness and drought resistance are correlated and that a quantitative determination of the former property might therefore serve as a measure of the latter. It was one purpose of the present work to test this suggestion.

MATERIALS AND METHODS

Leaves and seedlings from various strains of loblolly pine (*Pinus taeda* L.), whose drought resistance was well established by field experiment, were generously provided by the Texas Forest Genetics Institute. These strains, which were designated by the following symbols in decreasing order of drought resistance: BA-5, FA-2, AN-3, BA-2, LA-1, LA-2, TY-4, and NC, were shipped to the author's laboratory at different seasons of the year.

From the beginning of the experiment with pine leaves it was realized that, in contrast to sugar cane, it would be practically impossible to obtain drip from pine leaves by any direct or indirect method. Attention was directed to other tests suggested directly or indirectly by the literature on the subject of drought hardiness (9, 6).

Several attempts were made to correlate drought resistance with respiration intensity, with pentosan, calcium, protein, and ash content of the leaves. None of these studies gave results which were significantly correlated with drought hardiness.

It was thought that, if the ill effect produced by drought on the plants could be reproduced in the laboratory on parts of the plants (leaves, for instance) under standardized conditions, it might be possible to measure critical changes and to use them as a test for drought resistance. The first change observed on plants suffering from drought was the wilting of the leaves and the gradual fading of the green color. When the color change reached some critical point, recovery no longer was possible. An attempt was made to reproduce these changes by putting pine needles in large desiccators containing saturated solutions of various salts, placed at different temperatures. These test proved unsatisfactory, as did other subsequent tests with a desomatic dehumidifier and a constant temperature humidity chamber.

Attention was then turned to pigment changes induced by heating which were found to correlate well with drought resistance. When pine needles were immersed in water and heated gradually in a temperature regulated water bath, a temperature was reached beyond which chlorophyll destruction proceeded rapidly. The critical temperature proved to be between 55 and 56° C.

A 5-g representative sample of healthy unblemished pine needles was placed in a large tube one inch in diameter with 50 ml of distilled water and heated in a water bath at 56° ± 1° C for exactly 30 minutes. At the end of this time the leaves were removed from the tubes and were ground in a Waring blendor for five minutes with 100 ml of 80 % acetone solution in water. The chlorophyll extract was filtered and the filtrate examined immediately for light absorption with a Klett photoelectric colorimeter using a no. 66 red filter. Pigment was extracted from a 5-g sample of unheated leaves and the light absorption was also measured. The difference between the two readings was defined as the "Chlorophyll Stability Index" (C.S.I.).

Other methods used in this study were: the determination of bound water as described by Nelson and Hulet (10) and the determination of sulfhydryl groups by the nitroprusside colorimetric procedure (1).

TABLE I

RELATION BETWEEN THE CHLOROPHYLL STABILITY INDEX OF PINE NEEDLES AND DROUGHT HARDINESS OF TREES FROM THE SAME REGION *

STRAIN	RELATIVE DROUGHT HARDINESS IN DECREASING ORDER **	COLOR READING WITH FILTER NO. 66		
		BEFORE HEATING	AFTER HEATING	DIFFERENCE (C.S.I.)
FA-2	1st	288	275	12
AN-3	2nd	284	260	24
BA-2	3rd	269	233	36
LA-1	4th	282	246	36
TY-4	5th	313	273	40
NC	6th	288	244	44
			Average difference	*32*

* Region C, Texas.
** From field observations.

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Results

Needles from the above mentioned strains of loblolly pine grown in five different regions of Texas were examined with the above procedure. The chlorophyll stability indexes (C.S.I.) of the various pine strains were found to correspond very closely to the drought resistance values obtained from field observations. The data of table I are typical of the general agreement between C.S.I. and resistance to drought. A summary of all such results appears in table II.

Critical conditions which induced pigment changes were those which might correspond to protein denaturation. It is known that the amount of bound water and the presence of free sulfhydryl groups are related to protein denaturation (7, 8). The data of table III indicate that values of bound water or of extractable sulfhydryl groups might be worked out on a statistical basis to provide material which could be used as a test for drought resistance. The reliability of these tests perhaps will be somewhat lower than that of the chlorophyll stability index (C.S.I.). The detailed mechanism of the process of pigment change and the products of chlorophyll degeneration are not known. Regardless of mechanism, the test using the C.S.I. is of empirical value and may be used to predict drought resistance in pine.

Summary

A laboratory test for drought resistance has been developed based on the heat stability of chlorophyll in situ. The test was tried with pine strains of known resistance to drought and a good correlation between chlorophyll stability and drought resistance was found. It appears to be a more reliable test of drought resistance than bound water or extractable sulfhydryl groups.

Table II

Deviations of Chlorophyll Stability Index from the Average Value *

Strain	Relative drought hardiness in decreasing order **	Region where material was collected				Departure from average (C.S.I.)
		A	B	C	D	
BA-5	1st	−16				−16
FA-2	2nd	− 9	− 6	−20	− 3	− 9
AN-3	3rd	..	..	− 8	..	− 8
BA-2	4th	−12	− 5	+ 4	0	− 4
LA-1	5th	+ 3	0	+ 4	..	+ 2
LA-2	6th	+ 8	+ 1	..	..	+ 4
TY-4	7th	+15	+ 5	+ 8	+ 3	+ 8
NC	8th	..	+ 5	+12	+ 6	+ 8

* Summary of 4 sets of data obtained from 4 growing regions of Texas.
** From field observations.

Table III

Relation Between Drought Hardiness, Bound Water, and Extractable -SH Groups *

Pine strain	Relative drought hardiness in decreasing order **	% Moisture ***	% Bound water ***	-SH groups *** (Color reading at $m\mu$ 520)
BA-5	1st	60.16	5.83	93
FA-2	2nd	62.08	6.07	81
AN-3	3rd	64.08	5.11	79
BA-2	4th	64.53	5.49	74
LA-1	5th	59.53	5.32	68
LA-2	6th	59.50	4.19	70
TY-4	7th	61.13	4.20	65
NC	8th	61.82	3.97	66

* 2 g of leaves heated to boiling in 25 ml water for 30 min before applying the nitroprusside test.
** From field observations.
*** Average of 4 determinations.

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Literature Cited

1. Glick, D. Methods of Biochemical Analysis. Vol. I. P. 21. Interscience, New York 1954.
2. Kaloyereas, S. A. Drip as a constant for quality of frozen foods. Food Research 12: 419–428. 1947.
3. Kaloyereas, S. A. Strawberry studies. A new method for determining firmness. Fruit Products Jour. 27: 6–7. 1947.
4. Kaloyereas, S. A. Use of dry ice and carbon dioxide for food preservation. Refrig. Eng. 57: 453–463, 556. 1949.
5. Kaloyereas, S. A. L'exaudat comme "test" de la qualité pour les aliments congelés. Un test de laboratoire pour la resistance au gel des plantes. Proc. IX Intern. Cong. of Refrig. Paris, 1955. Refrig. Abstracts 11: 100[4]. 1956.
6. Levitt, L. The Hardiness of Plants. Academic Press, New York 1956.
7. Littman, F. E., Carr, E. M. and Clauss, J. K. Protection of sulfhydryl groups against ionizing radiation. Science 125: 737–738. 1957.
8. Lundgren, H. P. Discussion of theory of molecular interaction in proteins and possible applications in food technology. Food Technol. 1: 70–77. 1947.
9. Maximov, N. The Plant in Relation to Water. Macmillan, New York 1929.
10. Nelson, O. A. and Hulet, G. A. Jour. Indus. Engin. Chem. 12: 40–45. 1920. (See also Gortner, R. A. Selected Topics in Colloid Chemistry. Pp. 148–150. Cornell University Press, Ithaca 1937.)

American Society of Plant Physiologists, *Southern Section:* The Southern Section of the American Society of Plant Physiologists met with the Association of Southern Agricultural Workers at Little Rock, Arkansas, in February 1958. The attendance was one of the largest in the history of the Section with a two-day program of interesting papers followed by the Annual Plant Physiologists breakfast at which Dr. L. M. Rohrbaugh, University of Oklahoma, spoke on "Effect of Growth Regulators on Plant Metabolism."

Symposiums on "Gibberellins" and "Recent Research on the Nutrition of Plants" highlighted the programs. Dr. S. B. Hendricks, Vice-president of ASPP was leading speaker of the latter.

New officers elected to take office at the close of the meeting were: Chairman, Aubrey W. Naylor, Duke University; Vice-chairman, Howard J. Teas, University of Florida; and Secretary-Treasurer, Robert E. Burns, USDA, ARS, Experiment, Georgia.—Robert E. Burns, *Secretary-Treasurer.*

Traité de Chimie Biologique, Vol. I—Louis Genevois. Presses Universitaires de France, Paris. 325 pages. 1957. 1800 F.

This volume, the first of a two-volume set on biological chemistry, is part of the "Euclid" series of elementary treatments of various scientific subjects. In content it greatly resembles books of this country's "College Outline Series" in that a great many items are covered, but only a small amount of basic information is presented on each item. The present volume discusses the chemistry and metabolism of amino acids, proteins, nucleic acids, porphyrins, and other nitrogenous compounds. Although information concerning these substances (especially their chemical properties) is well-chosen and clearly presented, a few questionable statements appear in the book. Thus, we are told on page 1 that animals do not have the ability to form 11 of the 18 amino acids found in their protein. Actually this number varies between 8 and 13 in different animals. On page 39 the Krebs cycle is depicted as beginning with a condensation of pyruvate and oxalacetate to form an ill-defined mixture of aconitate, isocitrate, and citrate. Most biochemists abandoned this concept following the demonstration of Ochoa and coworkers of the condensation of acetyl-CoA and oxalacetate to form citrate. On page 219 it is stated that asparaginase hydrolyzes asparagine and glutamine. Although asparaginase has not been purified completely as yet, the reviewer knows of no purified preparation that has any ability to hydrolyze glutamine. These questionable statements, however, are more than offset by the surprising amount of useful information to be found in this book, especially regarding the characteristics of substances of biological interest. Although the book is too elementary for students specializing in biochemistry, it could be a useful biochemical reference for students in other areas of biology.—George C. Webster, *Biochemical Laboratories, The Ohio State University, Columbus, Ohio.*